THE METABOLISM OF CARDIAC GLYCOSIDES

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THE METABOLISM OF CARDIAC GLYCOSIDES

A Review of the Absorption, Metabolism and Excretion of Clinically Important Cardiac Glycosides

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INTRODUCTION

THE cardiac glycosides, particularly those derived from species of digitalis, have been used extensively in the treatment of cardiac disorders for over two hundred years. During the past forty years a great deal of research work has been carried out on their clinical applications and upon their effect on cardiac tissue, but there is still a great deal to be learned about their pharmacological properties. It is only in comparatively recent years that relatively pure glycosides have become available to make possible any worthwhile assessment of the pharmacological differences of the glycosides. Furthermore the lack of sensitive methods of separating and estimating the glycosides made it extremely difficult to obtain accurate measurements of absorption, body distribution or of excretion rates. With the development of sensitive methods of biological assay, such as the embryonic chick heart, and the preparation and use of radioactive glycosides, the quantitative estimation of extremely small quantities of these substances in animal tissues became possible. The application of paper chromatographic techniques has also made it possible to study qualitatively the transformations undergone by the glycosides *in vivo*. Whereas it was always assumed that the original glycosides were distributed throughout the tissues and excreted unchanged in the urine, it is now possible to detect the presence of cardioactive metabolites in tissues and excretory fluids and measure them quantitatively.

The pharmacological background to the therapeutic use of cardiac glycosides has been reviewed by Rothlin and Bircher[illegible] while Friedman *et al*[illegible] have reviewed the fate of digitoxin in animals and humans. It is the purpose of this review to compare the behaviour of the important glycosides used in therapeutics, particularly in respect to their metabolic breakdown and excretion, and to review the salient facts upon which are based present concepts of the relationship between their chemical constitution and pharmacological activity.

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THE METABOLISM OF CARDIAC GLYCOSIDES

Chapter I

THE CHEMICAL NATURE OF CARDIAC GLYCOSIDES

THE term *cardiac glycoside* is applied to a group of naturally occurring substances possessing basically similar actions and chemical structure. Representatives of the class are found in a number of botanical sources, but those important clinically are derived from the families Scrophulariaceae, Apocynaceae and Liliaceae.

The chemistry of cardiac glycosides has been adequately reviewed in recent years by Turner,[164] Heusser,[75] Reichstein,[134] Shoppee and Shoppee,[161] Stoll and Renz,[155] and Tamm.[159] It is only necessary here to draw attention to the chief structural features of the clinically important glycosides. The following are the minimum requirements for functional groups.

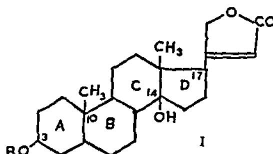
1. The genins are derived from the cyclopentanoperhydro-phenanthrene nucleus characteristic of steroid compounds (Formulae I-V).

2. At carbon atom 17 of ring D of the nucleus is attached a five-membered unsaturated lactone ring (Digitalis-Strophanthus type) or a six-membered doubly unsaturated lactone ring (Squill type.)

3 Carbon atom 3 in ring A possesses a hydroxyl group which in the glycosides is joined by an ether linkage to one or more sugar residues of varying complexity. Many glycosides have common aglycones and differ only in the nature or number of the sugars present.

4. A hydroxyl group is always substituted at carbon atom 14 in the aglycone nucleus.

These structural features and differences conditioned by the various sugar components are illustrated in Formulae I-V.

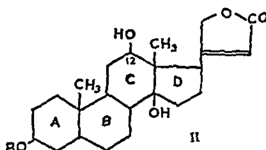


Lanatoside A (Digilanide A): R = (digitoxose)$_3$-D- glucose + acetyl. (Naturally occurring in *Digitalis lanata, Digitalis ferruginea.*)

Desacetyllanatoside A (Purpurea Glycoside A): R = (digitoxose)$_3$-D-glucose. (Naturally occurring in *Digitalis purpurea* or from Lanatoside A by hydrolysis with mild alkali.)

Acetyl Digitoxin: R = (digitoxose)$_3$+acetyl. (Derived from Lanatoside A by enzymatic action.)

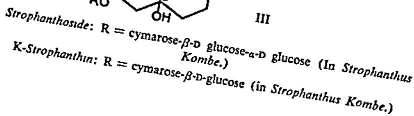
Digitoxin: R = (digitoxose)$_3$. (In *Digitalis purpurea* and *Digitalis lanata* by enzymatic breakdown of Purpurea Glycoside A or Lanatoside A.)



Lanatoside C. R = (digitoxose)$_3$ -D- glucose + acetyl. (Naturally occurring in *Digitalis lanata.*)

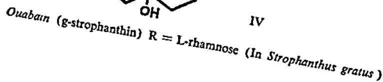
Desacetyllanatoside C: R = (digitoxose)$_3$ -D- glucose (Derived from Lanatoside C by hydrolysis with mild alkali.)

Digoxin R = (digitoxose)$_3$. (In *Digitalis lanata* by enzymatic breakdown of Lanatoside C.)

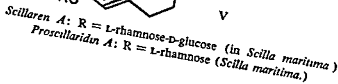


Strophanthoside: R = cymarose-β-D glucose-α-D glucose (In *Strophanthus Kombe.*)

K-Strophanthin: R = cymarose-β-D-glucose (in *Strophanthus Kombe.*)



Ouabain (g-strophanthin) R = L-rhamnose (In *Strophanthus gratus*)



Scillaren A: R = L-rhamnose-D-glucose (in *Scilla maritima*)

Proscillaridin A: R = L-rhamnose (*Scilla maritima.*)

THE STEREOCHEMISTRY AND CONFORMATION OF THE CARDIAC GLYCOSIDES

Theoretically the cyclic nucleus of four rings present in the cardiac glycosides could exist in a number of isomeric forms, since the component rings may be joined together by either *cis* or *trans* linkages as in the *cis* and *trans* decalins shown in Figure 1.

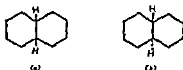


Figure 1. (a) *cis*, and (b) *trans* decalins.

However, the cardiac glycosides, unlike all other naturally occurring steroids have rings C and D always arranged *cis* to one another. *Rings B and C are always trans as in all steroids,* but rings A and B although normally *cis* (coprostane configuration) are in some glycosides arranged *trans* (cholestane configuration). Thus the aglycone digitoxigenin would be illustrated as in Figure 2 where heavy lines indicate orientation above the plane of the molecule.

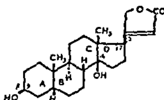


Figure 2. Stereochemical configuration of Digitoxigenin *Note*: Groups above the plane of the ring system are called β orientated and their valency bonds are drawn with a thick line. Groups below the plane are called α and the valencies are drawn with a broken line.

Other centres of specific interest are at C_3 where the —OH group is above the plane of the molecule (β orientated) and at C_{17} where the lactone ring is also β orientated. The effect of changing the spatial arrangement of these centres will be discussed in the following section dealing with the relationship between chemical constitution and pharmacological activity. It should be noted that the C/D *cis* arrangement, peculiar to cardiac glycosides, appears

to be conditioned by the presence of the hydroxyl group at C_{14} which is not found in other naturally occurring steroids.

In order to appreciate fully the stereochemical features of these molecules, it is necessary to consider the arrangements in space or *conformation* of the atoms which may be produced by rotation or twisting (but not breaking) of bonds. This modern concept of stereochemistry is based on the work of Hassel and his collaborators[46] and the conformation of the steroid nucleus has been ably discussed by Barton[3] and Klyne.[33]

Models of the molecules of cyclohexane show that it can exist in two spatial forms known as the "chair" and the "boat" forms (Figure 3).

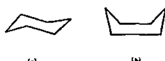


Figure 3. Cyclohexane: (a) chair form, and (b) boat form.

It has now become established that the "chair" form is the more stable of the two, and the most stable conformation of a fused cyclohexane system such as is found in the steroids is that which has the maximum number of "chairs." In the chair conformation of cyclohexane, two types of spatial arrangements of the C-H bonds can be distinguished. Those which are perpendicular to the general plane of the molecule and known as "axial" and those which lie approximately in the plane of the molecule are said to be "Equatorial" (Figure 4).

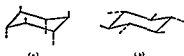


Figure 4 (a) Axial bonds of cyclohexane. (b) Equatorial bonds of cyclohexane.

This arrangement of C-H bonds can play an important part in the stability of the molecule, or more particularly in the stability of the substituents in the carbon atoms of the nucleus. The equatorial arrangement is in general thermodynamically more stable

than the axial. Thus if there is a choice as to whether an entering group may assume either the equatorial or axial position (as for example the hydroxyl group in the reduction of a C=O group to > CHOH) the preference will be for the equatorial. Furthermore a substituent linked to the nucleus by an equatorial bond is less sterically hindered than the same substituent linked to the same carbon atom by an axial bond. Thus apart from questions of stability of the whole molecule, the effects of conformation will also be seen in the course and rate of reactions involving substituent groups.

The conformational arrangement of digitoxigenin is shown in Figure 5 (a) where the cyclohexane rings are all arranged in the chair form. The conformation of a cardiac aglycone in which rings A and B are *trans* (uzarigenin) is shown in Figure 5 (b). The orientation of the substituents found in the important cardiac aglycones for both series A and B *trans* and A and B *cis* are given in Table I.

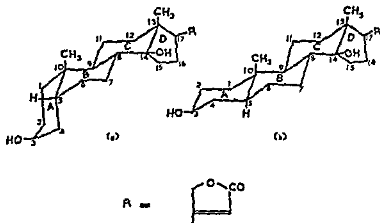


Figure 5. (a) Digitoxigenin A/B *cis*. (b) Uzarigenin A/B *trans*

The importance of stereochemical effects on the activity of cardiac glycosides will be outlined in the following section. Molecular conformations are more subtle forms of stereochemistry and their effect upon biological activity has not been evaluated. The steric arrangement of functional groups and the thermodynamic stability of the molecule could conceivably have some effect upon the way the molecule is attacked in the body, particularly in the way sub-

TABLE I

CONFIGURATION AND CONFORMATION OF SUBSTITUENTS IN CARDIAC AGLYCONES

Series	*Position*	α *config.*	β *config.*
A/B cis	3	e	a
or 5β	5	—	e^{B} a^{A}
	10	—	e^{A} a^{B}
	11	e	a
	13	—	a
	14	—	e
A/B trans	3	a	e
or 5α	5	a^{AB}	—

a = axial e = equatorial confirmation

e^{A} and e^{B} = equatorial to rings A and B respectively

stituent groups are removed or added. Much more knowledge of the enzyme systems involved must accumulate before the full significance of these factors can be evaluated.

Chapter II

THE RELATIONSHIP BETWEEN MOLECULAR STRUCTURE AND PHARMACOLOGICAL ACTIVITY

ALTHOUGH the chemical structures of the important cardiac glycosides have been established beyond reasonable doubt, the actual synthesis of a naturally occurring glycoside remains unaccomplished. Also no synthetic molecule which possesses cardioactive properties even approaching these of the plant glycosides has yet been prepared.

The synthesis of more highly active cardio-tonic substances is in itself not of great importance clinically unless accompanied by an increase in the therapeutic index (ratio of median toxic dose to median inotropic dose). Part of the difficulty in preparing substitutes with less toxic properties is due to the lack of precise knowledge of the true relationship between molecular structure and pharmacological activity. Part is also due to lack of knowledge of the mode of action and of the metabolism of the glycosides, and to the somewhat unsatisfactory nature of the methods of testing their activity. Reliance has been mainly placed on toxicity tests in gathering this information and these may or may not be of significance in relation to the failing heart. Some very important generalizations regarding structure-activity relationships have however been made and the evidence upon which these are found will now be examined.

CHEMICAL STRUCTURE

By far the greatest amount of data concerning the relationship of chemical constitution and pharmacological activity in this group of drugs has been gathered together by Chen and co-workers,[illegible] and has been summarized in reviews.

This group has examined 150 different cardiac glycosides and

derivatives. The results obtained from this extensive survey rely mainly upon the measurement of the cat lethal dose and assume that the toxic effect of the glycoside is an extension of its cardiotonic effect upon which the therapeutic action of the substance depends. In this method the glycoside is injected intravenously into the intact animal and circulates in the blood through all organs. Chen has also used both the frog lymph-sac toxicity method[illegible] and the toxic effect upon the isolated frog heart[illegible] in more limited surveys with, on the whole, the same result as found by the cat method. The results of these surveys are quoted widely in the literature and the conclusions drawn from this work can be used as a basis for a discussion of the problem. Some examples of lethal doses for a few typical glycosides or genins are shown in Table II.

TABLE II

LETHAL DOSES (CAT) OF SOME CARDIAC GLYCOSIDES AND AGLYCONES

	Cat Lethal Dose (mg/kg)
Digitoxin	0 325
Digitoxigenin	0 459
Digoxin	0.231
Digoxigenin	0 441
Lanatoside C	0.233
Ouabain	0.116
Strophanthin-K	0 128
Uzarin	4 58
Convallotoxin	0 079

THE LACTONE RING

It has been generally accepted that interference with the lactone ring results in a loss of toxicity. Hydrogenation of the double bond very greatly reduces if not abolishes cardiac activity. Thus Chen and Elderfield[illegible] found that dihydrostrophanthidin (Figure 6A) was almost completely inactive (65.8 mg/kg had no effect in cats whereas the lethal dose of strophanthidin was 0.325 mg/kg) and Jacobs and Hoffman[illegible] found that dihydrocymarin was about 1/20 and dihydro-ouabain about 1/16 times as active as the respective glycosides when tested by frog toxicity methods.

The unsaturated lactone ring must also be attached in the β-configuration at C17; epimerisation of the lactone ring as in allocymarin and allostrophanthidin reduces their pharmacological activi-

ty to less than 1/400 and 1/192 of the activity of the parent glycosides respectively (Chen and Elderfield,[33] and Jacobs[77]). Opening the lactone ring by alkaline hydrolysis also results in complete loss of activity (Chen[31]).

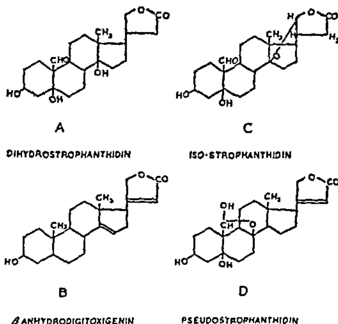


Figure 6

Vick, Kahn and Acheson[144] have recently used the dog heart-lung preparation (Farah and Maresh[50]) to determine the ratio between the toxic and tonic activities of dihydro-ouabain, dihydrodigoxin and dihydrodigitoxin and compared them with the original glycosides. The decrease in atrial pressure and increase in cardiac output was taken as an index of the tonic activity of the glycoside, and the first appearance of cardiac irregularities as the indication of toxic activity. Dihydro glycosides were found to produce a therapeutic action at a lower percentage of their lethal doses than did the unsaturated glycosides and this was taken to indicate that the toxic effect of the glycoside was reduced more than the tonic effect by reduction of the lactone ring. Wollenberger[149] on the other hand stated that hexahydroscillaren was completely inactive on the heart-

lung preparation. Stafford[illegible] found that dihydrodigoxin possessed very similar properties to digoxin with about 1/100 of its potency on the guinea pig Langendorff heart, and that the first signs of electrocardiograph irregularity produced in the cat by dihydrodigoxin were quite typical of the parent glycoside This work of Vick, *et al.*[illegible] will therefore need further verification, but it indicates the necessity to evaluate cardiac glycoside action on other criteria besides toxicity.

THE 14-BETA HYDROXYL GROUP

This hydroxyl group appears in all active cardiac glycosides. When it is removed, as in the formation of anhydrogenins, there is a loss in toxicity (Chen,[illegible] Tschesche and Snatzke[illegible]) (Figure 6B). Furthermore, if the 14-hydroxyl group is involved in a linkage with the lactone ring, an inactive compound is produced as seen in isostrophanthidin (Chen and Elderfield[illegible]) (Figure 6C). In this compound however, the lactone ring has also become saturated. Pseudostrophanthidin (Figure 6D) also has a low cat toxicity (66.7 mgm per kg) In this substance the 14-hydroxyl group has shifted to position 8 and become involved in a 6-membered lactol ring with the C-10 aldehyde group.

STEREOCHEMISTRY OF THE STEROID NUCLEUS

The cardiac genins are the only naturally occurring steroid compounds possessing a *cis* arrangement of rings C and D. When the 14-hydroxyl group is removed, as in an anhydrogenin (Figure 6B) this stereochemical relationship is destroyed so that loss of toxicity could be due either to the loss of the 14-hydroxyl group, or to the alteration of the *cis* C/D ring arrangement As yet no cardiac genin, either natural or synthetic has been found or prepared in which rings C and D are arranged *cis* without a hydroxyl group being present in position 14. It is therefore not possible to state at present whether the 14-hydroxyl group *per se* is necessary for activity, or whether the *cis* arrangement of rings C/D and hence the shape of the molecule is mainly responsible for potency.

The stereochemistry of ring A/B is normally the *cis* arrangement, and for a long time it was thought that this was critical for activity, as the aglycone of the glycoside uzarin (which has a low cat L.D. of 4.58 mg/kg) has always been held to have a *trans* A/B junction (Figure 7A). However, recent work has shown that cor-

otoxigenin and coroglaucigenin (Figure 7B) both have a *trans* A/B arrangement (Hunger and Reichstein) yet their respective glycosides, gofruside and frugoside, have L.D. cat toxicity levels of 0.19 and 0.161 mg. per kg. respectively, indicating that they are more toxic than digitoxin. Furthermore, it should be pointed out that rings A and B exhibit no stereochemistry in the active glycoside scillaren as there is an unsaturated linkage between carbon atoms 4 and 5 (Formula V p. 5.). Perhaps the structure of uzarigenin needs more critical examination.

OTHER HYDROXYL GROUPS IN THE GENINS

All cardio-active genins possess a hydroxyl group at C3. This is usually arranged in the beta position and in fact, Chen observed that conversion of digitoxigenin to 3-epi-digitoxigenin and tanghinigenin to 3-epi-tanghinigenin results in complete loss of activity. This is somewhat surprising in view of the fact that the genin of the glycoside urezin appears to be 3-epi-uzarigenin (Figure 7C) and this glycoside is slightly more toxic than uzarin. Also it has been reported (Chen and Henderson) that 5-anhydroperiplogenone has a digitalis-like action and that 3-scillarenone also possesses activity (Stoll, Renz and Brack). It is of course possible that in these molecules the 3-keto group is reduced to hydroxyl by the animal — a common reaction in the metabolism of steroidal hormones. Other nuclear substituted hydroxyl groups are found at C1 (ouabagenin), C5 (strophanthidin), C11 (sarmentogenin), C12 (digoxigenin) and C16 (gitoxigenin). Whilst having some modifying action, these nuclear hydroxyl groups do not appear to affect the toxicity to any marked extent so that they cannot be regarded as essential for activity. The C10 methyl group may be oxidized to —CH_2OH (strophanthidol) or to —CHO (strophanthidin) without much effect on toxicity. Oxidation to —COOH (strophanthidinic acid) however does result in a significant loss of activity (Figure 7D).

INFLUENCE OF THE SUGAR RESIDUES ON TOXICITY

A detailed study of the influence of the sugars on toxicity has been made by Chen and co-workers From this work it appears that in general the genins are less toxic than the corresponding glycosides, although the reverse is seen in sarverogenin and its

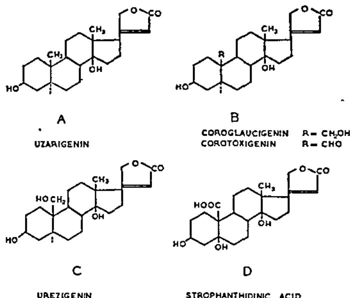


Figure 7.

glycosides intermedioside and pantroside, as well as in corotoxigenin and its glycoside milloside. The naturally occurring sugars do not necessarily produce the most toxic glycosides as their substitution by glucose often results in increased toxicity. There is some indication that the toxicity decreases with the addition of each monosaccharide unit, but this does not always follow since cheiroside A is more toxic than desglucocheiroside A[88]. Acetylation of the sugars produces variable results in toxicity and it is difficult to make useful deductions. It has been observed by several workers[143, 88] that the sugar residues of the glycosides diminish their effect upon the central nervous system. Digitoxigenin for example, produces convulsions in cats and frogs, whereas corresponding doses of the glycosides fail to produce similar effects.

It is well known that glycosides having 2-desoxy sugars linked to the genin are more easily hydrolyzed chemically than those with normal sugars. This does not however, have much bearing on toxicity, e.g., convallatoxin, which is the most toxic glycoside is a rhamnoside (L.D. cat 0.079 mg./kg)—but some rhamnosides are not as toxic as glycosides possessing the same genin combined with

2-desoxy sugars. Thus convalloside (strophanthidin+ramnose+glucose) is less toxic than k-strophanthin (strophanthidin + cymarose + glucose).

SIGNIFICANCE OF MOLECULAR VARIATIONS

The evaluation of the activity of cardiac glycosides by toxicity data and the conclusions drawn therefrom on the relationship between structure and activity are based on two assumptions:—

(a) That the cardiotoxicity of these molecules is but an extension of their cardiotonic properties, and

(b) That processes of metabolism do not produce alterations in the molecule resulting in an increase or decrease in toxicity or activity. This could apply especially to testing methods involving the use of intact animals.

The first assumption that the same mechanism is responsible for both the therapeutic and toxic actions of the cardiac glycosides is fairly widely accepted. In fact, toxicity results have invariably been the basis of potency estimations for clinical usage. However, there are methods of testing cardiac glycosides which depend upon their effect on a failing or hypodynamic heart. The papillary muscle preparation of Cattel and Gold is one such method, and these authors[39] found that a similar ratio existed between the therapeutic and toxic doses in a number of clinically important glycosides. White, Belford, and Salter[100] again using papillary muscle, obtained significant correlation between the cat lethal dose and inotropic response. Giarman[40] measured the cardiotonic effect of several glycosides and some naturally occurring lactones not containing the steroid nucleus, using frog hearts made hypodynamic by perfusion with low calcium Ringer. He considered that the basic mechanism of cardio-toxicity and cardio-activity was an expression of varying degrees of the same chain of chemical events. Farah and Maresh[41] using heart-lung preparations of the dog allowed to fail spontaneously or depressed with pentobarbitone, found that five glycosides could be assayed in the same descending order of potency when evaluated according to the "lethal" dose, "therapeutic" dose, or "toxic" dose. (However, as mentioned previously Vick *et al.*[108] using the same method of testing, claim to have shown that some dihydrocardiac glycosides have a more favorable therapeutic index than the parent glycosides). Bryan and Waldon[42] using the

embryonic chick heart obtained a good correlation between the therapeutic doses of a number of glycosides and the embryonic chick heart L.D. 50. Wollenberger and Karsh[155] measured the effect of cardiac glycosides on the contraction of guinea pig hearts inhibited by dinitrophenol and stated that the cardiac glycosides appeared to attack the same cellular enzyme systems in the therapeutic and toxic phases of their action.

However, even if it is conceded that the toxicity of these molecules which are known as cardiac glycosides, is an extension of their cardiotonic effect, it is still possible that other substances may be found which have a higher therapeutic index. Such substances could be regarded as having a more pronounced cardiotonic effect. The cardiac glycosides are not the only substances which have activity on the heart. The veratrine alkaloids do not resemble the cardiac glycosides in their chemical structure, yet they have some effects similar to the cardiac glycosides. The dilactone of pulvinic acid was reported by Giarman[60] to produce systolic standstill upon the isolated frog heart in the same order of concentration as that necessary for ouabain and digitoxin. Loynes and Gowdy[88] claimed a significant augmentation of hypodynamic frog hearts with 25 steroidal substances including cholesterol, ergosterol, calciferol and testosterone. Hajdu and St. Gyorgyi[83] reported that desoxycorticosterone exerts a cardiotonic effect upon frog's heart and Tanz and Whitehead[150] claimed that 9-*a*-fluoro-cortisone, cortisone and DOCA had a positive inotropic effect when tested on papillary muscle in concentrations of about 1 mg. per ml Emile and Bonnycastle[35] following up reports of the presence of cardiotonic substances in bovine and human serum, found that several adrenocortical steroids had a positive inotropic effect on papillary muscle. The activity of these substances was however much lower than that of 10 per cent serum when tested under the same conditions and is certainly much lower than that of cardiac glycosides.

The specificity of some of the pharmacological tests used to evaluate these substances leaves much to be desired, and in order to be more confident of their true cardiotonic effects, other methods of evaluation such as heart-lung preparations, need to be used. Nevertheless the possibility of finding potent substances differing in chemical structure from the cardiac glycosides should not be dis-

missed as entirely different action mechanisms and different enzyme receptors may be involved.

The second assumption that metabolic processes do not seriously interfere with the molecular structure of the glycosides needs critical examination. If stress is placed on slight structural alterations in the molecule before injection, then it is surely necessary to examine what happens to the molecule after it enters the body. Recent work to be discussed later has shown that cardiac glycosides do undergo important changes when they enter the blood stream and the significance of these changes must be considered.

Chapter III

METHODS FOR THE ANALYSIS OF CARDIAC GLYCOSIDES IN URINE AND ANIMAL TISSUES

CARDIAC glycosides may be estimated by biological and chemical methods, and both approaches have been used to measure the excretory rate and body distribution of the various glycosides. Earlier attempts to make quantitative measurements were handicapped by the lack of sensitivity of the methods available and also by the lack of specificity of colorimetric methods of chemical analysis before separation techniques had been devised. For biological estimation, toxicity responses on frogs, guinea-pigs, cats and pigeons were used, but relatively large quantities — fractions of a milligram to milligrams — are required for such methods. The lethal dose of digoxin for the cat is in the region of 230 mg per kg Nevertheless excretory or elimination rates of some glycosides have been measured with a reasonable degree of accuracy for example by Hatcher and Eggleston,[illegible] Farah and Smuskowicz,[illegible] Giertz, Hahn and Schunk.[illegible] The methods of biological assay in use up to 1950 have been reviewed by Loubatières.[illegible]

When microgram quantities or even fractions of a microgram have to be detected, much more sensitive methods than those outlined must be used. The minute quantities present in various tissues following administration of doses of the order of 0.5 to 1 mg. to an experimental animal can be detected only by using radioisotopes or very sensitive biological methods.

The use of carbon-14 digitoxin by Geiling and co-workers[100,101] has provided valuable information about the body distribution and excretion of digitoxin. The method is very accurate, and of course, very sensitive if the labelled glycoside can be separated from tissues

quantitatively uncontaminated by possible metabolites also containing carbon-14. This is not an easy task. The difficulties inherent in the purification of cardiac glycosides from plant materials are well known and hence any process of extraction from animal tissue must be well explored by rigorous control experiments with the labelled materials. Also, as pointed out by Friedman *et al.*[**] it is debatable whether a drug containing radioactive carbon should be administered to man.

SENSITIVE BIOLOGICAL METHODS FOR THE ANALYSIS OF CARDIOACTIVE MATERIALS

The biological methods available for estimating microgram quantities of cardiac glycosides are the papillary muscle preparation of Cattell and Gold[**] the isolated embryonic chick heart method of Lehman and Paff[**] and the isolated embryonic duck heart method of Friedman and Bine.[**] The papillary muscle preparation of Cattell and Gold is very sensitive (ouabain in dilutions of between 1 in 100 million and 1 in 5 million can be detected) but many substances in tissues, not cardiotonic, may also cause contraction of the fibre. It is therefore necessary to extract the glycosidic material quantitatively from tissues in a relatively pure state. The papillary muscle preparation is not easy to make quantitative, although White, Belford and Salter[***] were able to use it to obtain dose response curves and to compare the inotropic responses of a number of cardiac glycosides. The results obtained compared reasonably well with the cat toxicity methods. The inherent difficulties have prevented this method from being used in the study of the metabolism, distribution and excretion of cardiac glycosides.

The most successful biological methods for the study of the fate of the cardiac glycosides in animals and man are the embryonic duck heart or embryonic chick heart toxicity methods. Pickering[***] was the first to use embryonic chick hearts for detection of digitalis. Using embryos between 60-75 hours of age, he demonstrated the striking similarity of such hearts to those of adult mammals, especially in regard to their responses to *digitalis and strophanthus.* He found that the embryonic chick heart ceased beating after the injection of 0.012 mg. of digitalis *in situ*. Hall[**] employed the embryonic chick heart for the assay of crude digitalin, and Paff[***]

dissected the heart from the chick embryo and immersed it in the solution containing the drug to be tested. Using the occurrence of arrhythmia as a test for the presence of both ouabain and digitoxin, he was able to detect 0.01 mg. per ml. of ouabain and 0 001 mg. per ml. of digitoxin. The method was studied more intensively for assay purposes by Lehman and Paff.[66]

Friedman and Bine[44] used embryonic duck hearts for the detection of minute amounts of Lanatoside C. The embryonic duck heart was found to be about ten times more sensitive than the embryonic chick heart towards Lanatoside C which could be detected at a dilution of 1 in 20 million. The method was later applied to the estimation of digitoxin in mammalian urine, tissues and organs by Friedman and co-workers Although the duck heart is very sensitive to cardiac glycosides, relatively long times (20 to 40 minutes) are often needed for the observation of the heart block.

Wright and co-workers[130,9] used the embryonic chick heart to study the excretion of digoxin, digitoxin and Lanatoside C and their metabolites in rat urine. The technique of assay was that of Lehman and Paff[66] and no important modifications were introduced. By using a small cell with a volume of 0.05 ml. quantities as low as 0.005 microgram of digitoxin could be detected. This degree of sensitivity together with the ready availability of chick embryos makes the method a convenient one. Also by using extraction methods which reduce the toxicity of the urine extracts, it is possible to use times of from three to nine minutes to produce a block with chick embryo hearts, thereby speeding up the estimations.

It is necessary to incubate the eggs for a standard time of 48 hours before dissection, and to maintain the incubator temperature uniformly at 37-38 deg. C. The assays are conducted in a 2 + 2 arrangement using pure glycosides as standards so that an estimate of both the potency of the sample and a comparison of the slope of the dose response curve may be obtained This is a valuable check on the identity of the material being tested Groups of three hearts are used at a time and usually nine hearts are required to obtain any one point on the curve. Block times of from three to nine minutes are aimed at as the regression is linear when times of this order are used.

CHEMICAL METHODS OF ESTIMATION

Cardiac glycosides may be estimated quantitatively by measuring their absorption spectra in ultra-violet light. All cardiac glycosides having the butenolide ring structure at C-17 have an absorption maximum between 215 and 200 mμ (in most cases at 217 mμ) with a value of log E about 4.2. At such wave-lengths traces of impurities interfere, but if they can be eliminated the method is rapid and accurate. In extracts of animal tissues however the elimination of interfering substances is so difficult that the method is unsuitable.

There are a number of colorimetric methods available for the quantitative estimation of cardiac glycosides and several reviews have appeared.[18,121,80,182,182] Some of these methods can be applied to *urine and tissue extracts provided the glycosidic materials or their metabolites can be concentrated in sufficient quantity unaccom*panied by interfering substances. The application of paper chromatographic methods has enabled this to be done in many instances and this aspect of the problem will be discussed later.

In general the reagents used for colorimetric estimations are of two classes: (a) those of alkaline character which give colours with the butenolide ring, and (b) acidic reagents which react with the sugars or with the steroid portion of the molecule.

The alkaline reagents usually employed are: (1) alkaline m-dinitrobenzene (Raymond[107]). This gives an intense blue colour, maximum 620 mμ which fades rapidly. It may be used to estimate quantities of digitoxin as low as 20 microgm. if interfering substances are *easily eliminated. (2) 3,5-dinitrobenzoic acid* (Kedde[81]). Purplish colour, maximum 535 mμ which fades slowly. Slightly more sensitive than the Raymond reaction but interfering substances in tissue extracts modify the colour and limit its usefulness. (3) alkaline picrate (Baljet reagent modified Knudson and Dresbach,[77] *Bell and Krantz*[4]). *Sensitive to 5-10 microgm. of* digitoxin but many substances in tissues and urine extracts interfere. Other alkaline reagents which have been used for the estimation of pure glycosides or plant extracts are 2:4-dinitrodiphenylsulphone (Tattje[161]) and sodium β-naphthaquinone-4-sulphonate (Warren *et al.*[167]).

The acidic reagents. The most useful for quantitative estimation are those which give colours with 2-desoxy sugars. These are:

(1) Xanthydrol.[104,129,149] Gives a reddish colour stable for 20 minutes, maximum about 520 mμ and is about four times as sensitive as sodium picrate.

(2) Keller-Kiliani reaction (acetic acid-ferric chloride-sulphuric acid). Blue colour, maxima at 470 and 590 mμ. The modification of Lindewald[77] is suitable.

(3) Bial reaction used by Langejan.[87] Depends upon the colour given with orcinol in the presence of hydrochloric acid.

There are a number of acidic reagents which are used to assist in the qualitative detection of glycosides and which have a limited application only in metabolism work because of their relative non-specificity. Some of these are used for locating glycosides on paper chromatograms (p. 30). Concentrated sulphuric acid or 84 per cent sulphuric acid give characteristic colours with all glycosides and aglycones[140] Antimony trichloride and trichloracetic acid give colours with characteristic fluorescence in ultraviolet light with both glycosides and aglycones.

The glycosides derived from squill containing the doubly unsaturated six membered ring (bufadienolides) do not react with the reagents normally used for the digitalis-strophanthus group (cardenolides). They do however give the Liebermann reaction — a permanent blue colour with acetic acid — sulphuric acid — acetic anhydride, but this does not appear to have been adapted yet for the quantitative estimation of these glycosides in animal tissues.

The acidic reagents have a limited use in investigating metabolites of cardiac glycosides. Firstly a sugar residue capable of reaction must be present — and this normally means a 2-desoxy sugar. Thus they are limited to the digitoxose-containing glycosides and to metabolites which still retain these sugar residues Secondly the tissue or urine extracts must be fractionated to eliminate the wide variety of interfering substances. Volmar *et al.*[150] using the yellow colour produced by concentrated sulphuric acid on chloroform solutions of digitoxin, followed the excretion of this substance in guinea-pig urine without attempting to purify the cardioactive

materials other than by extracting the urine with chloroform. It is difficult to see how results based on such a non-specific reaction could be reliable.

Provided the cardioactive substances in urine or in tissue extracts contain a 2-desoxy sugar and can be concentrated in sufficient quantities by paper and column chromatography, xanthydrol is the most useful of these reagents. It may be used to develop a colour with the glycoside-containing areas of paper chromatograms without preliminary elution hence avoiding losses. This procedure was used by Wright and co-workers[40,41] for estimating digitoxin and digoxin and acetyl digoxin after separating the glycosides from bile and urine, and the following is an outline of the method.

The areas of the paper chromatogram containing the glycoside are cut into thin strips about 1 mm. wide and placed in a test tube. Then 20 ml. of xanthydrol reagent (0.125% xanthydrol in glacial acetic acid) are added followed by 0.1 ml. of concentrated hydrochloric acid. The tube is then placed in boiling water for 60 seconds and then immediately cooled by immersing the tube in ice and water. When cool a portion of the solution is transferred to a colorimeter tube and read against a control made with blank paper strips.

Repke[111] also used xanthydrol for estimating digitoxin in tissues and excreta after extraction with chloroform and purification by alumina chromatography. The sensitivity of the method was claimed to be 0.1 microgm. of digitoxin, but its main disadvantage was the use of strongly polar solvent mixtures (up to 10% in chloroform) to elute the glycosides from the column. Such mixtures are not likely to give clear separation of digitoxin and its metabolites.

The alkaline reagents have a wider application in cardiac glycoside metabolism studies. They will react with any glycoside or metabolite which still has the butenolide ring intact and as this grouping is essential for cardioactivity they may be used to follow and estimate the important excretory and metabolic products. The most useful of the reagents is alkaline metadinitrobenzene because the characteristic blue colour which it gives with the butenolide ring is unmistakable and not likely to be confused with the reddish-purple colour which the reagent gives with ketosteroids. The

main limitation is the transient nature of the colour, requiring quantitative estimations to be made under carefully controlled conditions. The following method is based on the procedure used by Anderson and Chen[1] and has been adapted for microgram quantities.

A solution of the material to be estimated is evaporated to dryness and the residue transferred to the colorimeter tube with 1.25 ml. of ethanol. To this is added 0.2 ml. of a 1% ethanolic solution of purified m-dinitrobenzene and the tube cooled thoroughly in an ice-water mixture; 0.4 ml. of 20% aqueous sodium hydroxide is then added and the contents of the tube mixed well by inversion. The tube is replaced in the ice-water mixture for *five* minutes and then read in a colorimeter using the appropriate filter within 30 seconds after removing from the ice bath. Standards are prepared and read under the same conditions.

Repke[111] also used m-dinitrobenzene for estimating digitoxin in tissue extracts and was able to obtain a stable colour after the addition of boric acid to the reagent mixture.

Radioactive digitoxin was first prepared by Okita, Kelsey, Walaszek and Geiling[119] by growing *Digitalis purpurea* in an atmosphere of carbon-14 enriched CO_2. The radioactive carbon dioxide was prepared from radioactive barium carbonate and during the 4 to 6 weeks growing period the labelled gas was injected into the chamber approximately once every six days.

The C-14 digitoxin extracted from the plant varied in specific activity from 0.36 to 0.56 microcuries per mg. and as little as 0.02 microgram of radioactive digitoxin could be assayed provided an internal gas flow counter was used. The advantages of the labelled glycosides are obvious and the results obtained by their use has been reviewed by Okita.[97] Minute amounts of the material can be detected and it is possible to distinguish between the original glycoside and its metabolites provided efficient separation methods are used.

Because of the small amounts available, it is not possible to use radioactive glycosides for the qualitative identification of possible metabolites. Once the metabolites have been identified by chemical or paper chromatographic means it may then be possible to use

the C-14 material for confirmation. This method proved useful in helping to identify digoxin as one of the metabolites of digitoxin.[illegible]

PAPER CHROMATOGRAPHIC SEPARATION OF CARDIAC GLYCOSIDES AND THEIR METABOLITES

Paper chromatographic methods have proved invaluable in investigating the metabolism of cardiac glycosides for detecting excretory products, for concentrating them prior to elution for quantitative estimation or for chemical studies, and for identifying the small amounts of cardioactive materials present in tissues, organs and excreta.

Because of the relatively large amounts of impurities extracted together with the glycosidic material whether it be from urine, blood or organs, it is necessary to use a system of paper chromatography which will permit heavy loading of the paper. Systems which depend upon the saturated water vapour of the tank for the stationary phase, e.g., the chloroform-methanol-water systems of Svendsen and Jensen[illegible] or the chloroform-ethyl acetate-benzene water systems of Silberman and Thorp[illegible] are of little use for the primary separation of metabolites from tissues, although they may be useful for subsequent identification of material eluted from other chromatograms.

The most successful methods of paper chromatography for this work are those in which an organic polar solvent such as formamide is used as the stationary phase. Such systems, first introduced by Zaffaroni and co-workers[illegible] for the separation of adrenocortical steroids, give excellent chromatographic resolution without "tailing" of the spots, yet permit heavy loading of the paper. Schindler and Reichstein[illegible] adapted the formamide systems to the separation of cardiac glycosides and many variations have since been used.[illegible] Strongly polar glycosides travel slowly on formamide impregnated paper, but it is possible to select a system which will give a preliminary separation of glycosides or metabolites from inert materials such as pigments and lipoid material, and then rechromatograph the eluted materials using different developing solvents and formamide impregnated paper.

Tschesche and co-workers[illegible] used reversed phase systems for the chromatography of cardiac glycosides in an attempt to overcome the adsorptive effects of the paper. In these methods the

paper is impregnated with the organic phase obtained after separation of mixtures of n-octanol, pentanol water and formamide and the aqueous phase used for development. These systems are useful for identifying eluates of glycosides or their metabolites obtained from formamide paper chromatograms because they are contrasting solvent systems in which the Rf values of the glycosides are reversed. Identification by paper chromatographic methods requires the use of at least two different solvent systems, and if possible the stationary phases should be different.

For the preliminary separation of glycosides and their metabolites in urine or tissue extracts, the following systems have proved satisfactory.

(a) Formamide impregnated paper with developing solvent chloroform 88 (vols) benzene 12 (vols) saturated with formamide. This system has been used for separating digoxin and its metabolites from chloroform extracts of urine.[10]

(b) Formamide impregnated paper with developing solvent chloroform 88 (vols) benzene 12 (vols) butanol 5 (vols) saturated with formamide. This is useful for the separation of the polar glycosides such as Lanatoside C[9] but it is also useful for digoxin metabolites in chloroform extracts of urine, blood, organs or bile.

(c) Formamide impregnated paper and developing solvent methyl isobutyl ketone 100 (vols) isopropyl ether 20 (vols) saturated with formamide.[11] This system is used for less polar glycosides such as digitoxin and acetyldigitoxin particularly when present in bile where excessive amounts of pigment must be carried ahead of the glycosides on the chromatograms.

Chromatography Technique. The following is an outline of the methods found most useful in this laboratory for chromatography of urine and tissue extracts on formamide impregnated paper.

Strips of paper (Whatman No. 4 or Schleicher-Schull 2043a) 30 cm. long and varying from 1.5 cm. to 5 cm. wide (depending upon the amount of extract) marked with a pencil line 5 cm. from one end are immersed in a freshly prepared mixture of formamide 25% or 30% in methanol or acetone for a few minutes. The proportion of formamide in this solution may be altered to increase or decrease the traveling distance of the glycosides. The strips are removed from the solvent, excess solvent drained off and then

exposed to the atmosphere to allow the volatile solvent to evaporate.

The chloroform extracts of the urine, tissues etc. are evaporated to dryness (reduced pressure) and redissolved in a small volume of chloroform-methanol (equal volumes). This solution is then streaked over the starting line using a capillary pipette over a current of warm air. Care must be taken to ensure even distribution of the extract across the paper and prevent excessive drying of the paper. The chromatograms are conveniently placed horizontally in a shallow glass tray (30 cm. x 20 cm. x 5 cm.) the top edge of which is ground flat and covered with a heavy glass sheet. A perfect seal is made by the use of bentonite glycerin paste or silicone grease. The bottom of the tank is covered to a depth of about 1 cm. with the developing solvent and the paper strips are supported on a glass rack 1 to 2.5 cm. high with the end near the starting line dipping into the developing solvent (Figure 8). The trays are kept at constant temperature, and an incubator regulated at a suitable temperature is convenient for the horizontal chromatograms and can accommodate many trays of the type described. Temperatures varying from 25 deg. to 37 deg. may be used depending upon room temperature but all solvent mixtures must be equilibrated at the selected temperature.

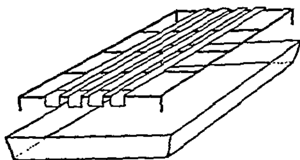


Figure 8. Glass tank and rack for horizontal paper chromatography.

After development the strips are dried in the atmosphere for a short time and either the entire strip or a longitudinal marginal area cut off from the strip (Figure 9.2b) is treated with a 5% solution of m-dinitrobenzene in benzene, allowed to dry, placed on a white slab and then flooded with 20% sodium hydroxide. A blue color appears immediately in the region where cardio-

active substances are present. The color fades quickly and facsimiles must be made on replicate paper strips. Shrinkage of the paper due to the sodium hydroxide is allowed for by previously marking the strip at intervals of 3 cm with a grease pencil.

The portion of the paper strip not treated with the reagents (Figure 9 2a) is then dried at 80° for 30 minutes and the areas corresponding to the glycoside or metabolite on the test strip can then be eluted with methanol and used for rechromatography on other systems or for quantitative analysis of biological methods (embryonic chick heart) or by colorimetry.

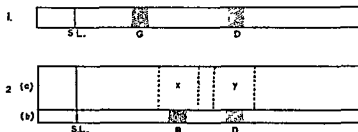


Figure 9 Paper Chromatograms of Chloroform Extract of Rat Bile after Digoxin Administration Formamide impregnated paper. Developing solvent —chloroform, benzene, butanol/formamide. (1) Control. (2) Bile Extract (a) = strip reserved for recovery of glycosides etc (b) Strip used for visualization. S L. = starting line D = digoxin. G = digoxigenin. B = *metabolite B*

In investigating the metabolism of digitoxin using radioactive carbon-14 digitoxin Okita *et al.*[161] used a system consisting of carbon tetrachloride-ethanol 95%-water 1:1:1 to identify digitoxin. The carbon tetrachloride rich layer was used as developing solvent and the alcohol-water was used to saturate the atmosphere in the tank. This system proved useful in identifying small amounts of digitoxin in extracts purified by column chromatography but would not be suitable for the primary separation of metabolites and glycosides directly from tissue extracts. Repke[114] reported the separation of several metabolites of digitoxin from extracts of organs, tissues and excreta of rats using the systems of Kaiser,[91] viz., xylene or benzene-methylethyl ketone (1:1) saturated with formamide, and chloroform-tetrahydrofurane-formamide

50:50:6.5. Both systems were used with formamide impregnated paper.

For the further identification of metabolites and glycosides Wright and co-workers[11,18] used the reversed phases systems of Tschesche *et al.*[111] and the chloroform benzene water systems of Silberman and Thorp.[118]

The most sensitive reagents for the detection of cardiac glycosides on paper chromatograms are the acidic reagents trichloracetic acid and antimony trichloride. Both are used in chloroform solution and the former is made more sensitive by the addition of an oxidizing agent such as chloramine[80] or hydrogen peroxide. The reagents have the advantage of giving different colors either in visible or ultraviolet light and this is often a guide in identification. The colors given with the commonly used glycosides are given in Table III.

These reagents are very sensitive and 0.5 microgram of the digitalis glycosides can be detected with trichloracetic acid. The colours produced are attributed to the steroid portion of the molecule and there are many substances in tissue extracts or in

TABLE III

COLOURS SHOWN BY DIGITALIS GLYCOSIDES AND GENINS IN VISIBLE AND ULTRA-VIOLET LIGHT AFTER TREATMENT WITH TRICHLORACETIC ACID-HYDROGEN PEROXIDE AND WITH ANTIMONY TRICHLORIDE

Substance	*Trichloracetic Acid (with added Hydrogen Peroxide)*		*Antimony Trichloride*	
	Visible	*U.V.*	*Visible*	*U.V.*
Lanatoside A	Grey-green	Yellow	Steel blue	Reddish purple
Acetyl digitoxin	"	"	"	"
Digitoxin	"	"	"	"
Digitoxigenin	nil	nil	"	Red
Gitoxin	Grey-green	Blue	Steel blue	Yellow
Gitoxigenin	nil	"	Pale blue	"
Lanatoside C	Grey-green	Pale blue	Violet	Pale yellow
Digoxin	"	"	"	"
Digoxigenin	"	"	Red-violet	Yellow

Colours Shown by Digitalis Glycosides and Genins in Ultra-violet Light on *Untreated Paper After Treatment* by Spraying with

(a) CCl_3COOH

(b) $SbCl_3$

urine which fluoresce with the reagents. The interpretation of chromatograms of crude extracts treated with these reagents may give misleading results. They are however of use in helping to identify materials which have been purified by paper or column chromatography.

The reagents of most use in this work are those which give colors with the butenolide ring of the aglycones. These are alkaline picrate, alkaline m-dinitrobenzene, and alkaline 2:4 dinitrobenzoic acid. Alkaline picrate is sensitive and rather less specific than the other reagents although Okita *et al.*[191] have used it to follow digitoxin on paper chromatograms of purified tissue and urine extracts.

Alkaline m-dinitrobenzene gives a very characteristic deep blue colour with most glycosides (exceptions are the squill group of bufadienolides) even in the presence of many contaminants and is not likely to be confused with the reddish pink colour given by ketosteroids. As the colour given is due to reaction with the butenolide ring only, those metabolites which retain this portion of the molecule intact will be detected. Metabolites without the butenolide ring would not appear to possess any cardioactivity. This reagent is less sensitive than trichloracetic acid or antimony trichloride and will not detect quantities of glycosides below about 5 micrograms on a chromatogram.

Chapter IV

CARDIOACTIVE METABOLITES OF THE DIGITALIS GLYCOSIDES

METABOLISM OF STEROIDS

THE METABOLISM OF STEROIDAL SUBSTANCES has been intensively studied in recent years and many reviews of this extensive subject have appeared (for current references see Callow[11] and Dorfman[12]). It is possible to classify the main metabolic reactions which result in modification of the intact steroidal nucleus under the following headings.

(a) Reduction of unsaturated linkages.
(b) *Reduction of carbonyl to hydroxyl.*
(c) Oxidation of hydroxyl to carbonyl.
(d) Hydroxylation.

The digitalis glycosides, the only cardiac glycosides whose metabolism has been studied in any detail, do not contain any unsaturated linkages or carbonyl groups in the steroidal nucleus, and there is no information about any reactions which may result in the attack of the unsaturated lactone ring. Interest, therefore, is centered mainly in the possibility of oxidation of hydroxyl groups or in hydroxylation reactions.

There are many examples of *in vivo* oxidation of hydroxyl groups in steroids. Thus the 11α hydroxyl group in corticosterone is oxidized to carbonyl giving 11-dehydrocorticosterone. Other examples are the oxidation of cortisone at C-17 to give 17-keto metabolites with a consequent loss of the chain thereby producing 19 carbon atom steroids. Testosterone and estradiol both undergo oxidation at C-17, the former giving androsterone and its isomer, the latter giving estrone. Examples such as these indicate the possibility of oxidation of hydroxyl groups occurring in the cardiac glycoside genins. Thus the C-12 hydroxyl group of digoxigenin

glycosides might yield 12-keto derivatives, or the oxidative removal of the C-17 unsaturated lactone ring might be expected. As yet no evidence of such oxidative reactions has been demonstrated in cardiac glycosides.

Hydroxylation of steroids is a very common metabolic reaction and usually results in the formation of more polar metabolites which are likely to be more water-soluble (or less soluble in lipids) than the parent compounds. Most positions in the steroid nucleus have been shown to undergo hydroxylation by mammals or by microorganisms (Grant[illegible]). In mammals the following positions have been shown to be hydroxylated: 2(α), 3(β), 6(β), 7(α), and (β), 11(β), 12(α) and (β), 16(α), 17(α), 18, 19, 20, 21. In addition to all of these positions, hydroxylation by microorganisms has been demonstrated at 8, 11(α), 14(α) and 15(α) and (β). It might therefore be expected that hydroxylation would be a likely method of metabolic attack in the digitalis glycosides, and this has been shown to occur. However very few positions in the steroid nucleus of the glycosides appear to be susceptible to hydroxylation by mammals or by microorganisms.

DIGITALIS GLYCOSIDES

It might be expected that cardiac glycosides would undergo hydrolytic fission in animals to produce the corresponding aglycones, and several earlier investigators, Fischer,[illegible] Weese[illegible] and Lendle,[illegible] reported the formation of digitoxigenin from digitoxin under various experimental conditions. Reliable chemical methods of identification, applicable to the very small quantities of materials involved, were not then available and no confirmation of the presence of free aglycones in animal tissues or excreta after the administration of glycosides has been obtained in recent years

The presence of metabolites of digitoxin in tissues, organs and excreta after the administration of radioactive (carbon-14) digitoxin has been reported by Fischer *et al.*[illegible] and by Okita *et al.*[illegible] Because of the small amounts of materials involved, these workers were not able to determine whether these radioactive metabolites were cardioactive, nor was any information obtained about their chemical nature.

Metabolites of the glycosides digitoxin, acetyl digitoxin, lanatoside C and digoxin which still possessed cardiac activity were first

mula II, p. 4) is apparently the precursor of digoxin in *Digitalis lanata*, and unless care is taken to inhibit enzymatic action during drying or extraction of this leaf it loses glucose and an acetyl group to yield digoxin. It behaves as a much more polar substance than digoxin on paper chromatograms and is also more water-soluble. In the rat and in man, lanatoside C is excreted mainly unchanged, but when large doses are given it is possible to detect digoxin in urine indicating that deacetylation and loss of glucose occur. Some *metabolite B* may also be detected at high dose levels indicating removal of the digitoxose residues followed by conjugation.[11] A typical paper chromatogram of a chloroform extract of rat urine after high dose levels of lanatoside C is shown in Figure 11B.

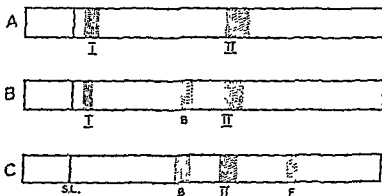


Figure 11. Paper chromatograms of extracts of rat urine after Lanatoside C and Digoxin administration. Formamide impregnated paper. Developing solvent:— chloroform benzene/formamide A. Control. B Extract of Lanatoside C urine. C. Extract of Digoxin urine. I = lanatoside C; II = digoxin; B = *metabolite B*, F = *metabolite F*

Digoxin (Formula II, p. 4) is excreted in rat urine and in human urine partly unaltered and partly as *metabolite B*[11,9] (Figure 11 C) The nature of *metabolite B* has been outlined previously in the discussion of the metabolites of digitoxin as the same metabolite is produced after digitoxin and acetyl digitoxin administration. *Metabolite B* is also excreted when digoxigenin is administered to rats. A second digoxin metabolite known as *metabolite F* is present in small amounts in rat urine after high level doses of digoxin (5-6 microgm. per gm.).[11] The nature of this substance is

unknown, but from its behaviour on paper chromatograms it is slightly less polar than digoxin (Figure 11 C).

The inter-relationship of the metabolites of the clinically important digitalis glycosides is shown in Figure 12.

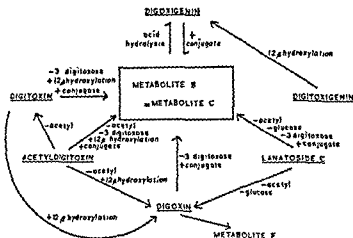


Figure 12.

THE METABOLISM OF DIGITOXIGENIN AND ITS DERIVATIVES BY MICROORGANISMS

Gubler and Tamm[11] have recently studied the metabolism of digitoxigenin by the fungus *Fusarium lini*, and found that hydroxylation also occurred in the 12β position to yield digoxigenin. The 3-acetyl derivative of digitoxigenin also produced digoxigenin under the same conditions thereby undergoing 12-β hyroxylation and deacetylation The fungus, however, was not able to hydroxylate the glycosides digitoxin, somalin (D-cymaroside of digitoxigenin) or evomonoside (L-rhamnoside of digitoxigenin). This difference between the behavior of digitoxigenin and digitoxin towards hydroxylation by the fungus contrasts with the ability of animals to hydroxylate both substances. When tetra-o-acetyl digitoxin was fed to *Fusarium lini*, deacetylation only occurred yielding digitoxin.

The oxidation product of digitoxigenin, 3-dehydrodigitoxigenin (Figure 13, IV) was also attacked by *Fusarium lini* to give three reaction products. The main metabolite was a reduction product,

3-epi-digitoxigenin (Figure 13, V) and this was accompanied by some 3-dehydrodigoxigenin and 3-epi-digoxigenin (Figure 13, VI and VII). Evidently this microorganism possesses enzymes capable of deacetylation (esterase), oxidation of the steroid nucleus and reduction of the ketone group.

The same fungus was also able to metabolize the steroids androsten-(4)-dione (3-17), testosterone, and progesterone. In these steroids position C-12 was not hydroxylated, but C-5 instead was almost exclusively attacked. Some hydroxylation also occurred at C-6. Evidently the 15 and 6 positions are preferred but the presence of the large lactone ring in the cardiac glycosides at C-17 prevents attack at C-15 and the fungus instead

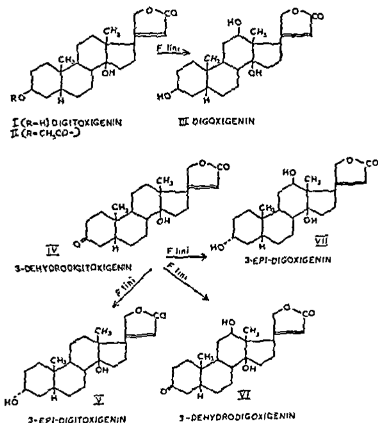


Figure 13. Metabolism by digitoxigenin and derivatives by *Fusarium lini*.

hydroxylates at C-12. The presence of the $\Delta^4$3-keto grouping in ring A of these steroids would also tend to activate position 6 and this would explain why no C-6 hydroxylation has yet been observed in cardiac glycosides.

Chapter V

THE ABSORPTION OF CARDIAC GLYCOSIDES

THERE SEEMS TO BE A GENERAL agreement that digitoxin is almost completely absorbed from the gastro intestinal tract of most animals including man. The evidence for this is drawn from many sources and has been conveniently summarized by Friedman, St. George and Bine.[45] The guinea pig seems to differ in this respect as Hotovy[77] found that absorption did not appear to be complete in this animal. Cats absorb digitoxin completely and Rothlin and Bircher[119] consider that it is the most suitable animal for the determination of the absorption of glycosides from the gastro-intestinal tract. Closely comparable toxic doses have been arrived at by intravenous and intraduodenal infusion in the hands of several investigators.

Rothlin and Schalch (reported by Rothlin and Bircher[111]) determined the dose required to produce heart block for a number of glycosides by administering them to cats by slow intravenous and intraduodenal infusion. From the relative lethal doses an "intestinal absorption quotient" was calculated and the values obtained are shown in Table IV. It will be noted that low ratios were obtained for the glycosides acetyl-digitoxin, digitoxin and acetyl digoxin, indicating a high degree of enteral absorption. Digoxin was not as completely absorbed as this group, while lanatoside C and lanatoside A showed less absorption still. The glycosides lanatoside A, desacetyl lanatoside A, desacetyl lanatoside C, k-strophanthin and ouabain gave very high ratios and apparently undergo little or no absorption from the intestine. In fact, with the last group heart block could not be produced by intraduodenal infusion, and to obtain an approximate value for the "absorption quotient" of these glycosides an intravenous infusion (of the standard Hatcher type) was given immediately after the termination of

the unsuccessful duodenal experiments and values of between 40-75% of the normal Hatcher values were obtained. The amount of the glycoside administered intraduodenally was divided by the difference between the "normal Hatcher dose" and the "intravenous filling dose" to give an approximate enteral absorption quotient. Values arrived at by this method are very approximate as allowance is not made for such factors as inactivation in the duodenum or elimination and metabolism during the slow intraduodenal infusions.

TABLE IV

RATIO BETWEEN INTRAVENOUS AND INTRADUODENAL TOXIC DOSES FOR CARDIAC GLYCOSIDES IN CATS. (INTESTINAL ABSORPTION QUOTIENT)

Glycoside	*Number of Free Hydroxyl Groups in Steroid Nucleus*	*Sugars Present*	Ratio* *I.V. and I.D.*
Lanatoside A	1	glucose + digitoxose (+ acetyl)	1:~5
Desacetyle-Lanatoside A	1	glucose + 3 digitoxose	1:~9
Acetyl digitoxin	1	3 digitoxose (+ acetyl)	1:1.5
Digitoxin (Sandoz)	1	3 digitoxose	1:1.33
Lanatoside C	2	glucose + 3 digitoxose (+ acetyl)	1:5.6
Desacetyl-Lanatoside C	2	glucose + 3 digitoxose	1:~20
Acetyl digoxin	2	3 digitoxose + acetyl	1:1.8
Digoxin	2	3 digitoxose	1:2.3
k-strophanthoside	2 + —CHO	cymarose + 2 glucose	1:~20
Ouabain	5	rhamnose	1:~11

* After Rothlin and Schalch reported by Rothlin and Bircher[119]

These results support clinical findings that the glycosides lanatoside A, desacetyl lanatoside A, desacetyl lanatoside C, k-strophanthin and ouabain are unsuitable for oral usage. Rothlin and Bircher[119] recommended that this method of slow intraduodenal infusion be used for all comparisons of the intestinal absorption of glycosides.

One of the important constituents of *Digitalis lanata* and *Digitalis purpurea*, namely, gitoxin is apparently either not absorbed at all from the gastrointestinal tract or is inactivated there because of allomerisation through the hydroxyl group in C-16 (Hotovy[illegible]). It is however active by intravenous injection. For this reason it is

difficult to translate potencies for galenical preparations or extracts of digitalis arrived at by intravenous dosage into terms of oral doses.

White and Gisvold[101] also studied the absorption of several glycosides by giving oral doses intended to kill cats and observing the survival times. They found the following ratios of oral to intravenous doses. Digitoxin 1.05; acetyl digoxin 1.15; digoxin 1.6. Because of its low absorption under the experimental conditions a value for lanatoside C could not be calculated. They also found that acetyl digoxin, digoxin and digitoxin were more rapidly absorbed from the gastrointestinal tract if the glycosides were administered in alcohol solution instead of in tablet or capsule form. The addition of a surface-active agent (Tween 80) also appeared to have some effect in promoting more rapid absorption.

From these results and the data given in Table IV there appears to be a relationship between the chemical structure of the glycosides and the degree of their absorption from the gastrointestinal tract of the cat. If digitoxin is taken as the most readily absorbed glycoside the presence of additional hydroxyl groups in the genin or of extra sugar residues, particularly glucose, appears to reduce absorption. An increase in hydroxyl groups or of sugar residues produces a more polar molecule and this may result in increased water solubility or decreased solubility in tissue lipids. The water solubility of some of the glycosides under discussion often shows little variation, e.g., digitoxin is soluble about 0.014 mg. per ml. and digoxin about 0.015 mg. per ml. A more positive correlation between structure and degree of absorption could be looked for in the lipid solubility of the glycosides for which there is no published data. A similar type of relationship between molecular polarity and biliary excretion has been observed and this is discussed in Section VII of this review.

Clinical evidence for the absorption of cardiac glycosides by humans shows a similar picture to the results obtained in the cat. Digitoxin, as indicated previously, is more than 90 per cent absorbed. A comparison of the maintenance dose of lanatoside C by intravenous and oral routes has been made by Hedland (reported by Rothlin and Bircher[111]). It was found that 0.4 mg. by intravenous injection was equivalent to 1.0 mg. orally, a quotient

of 1:2.5, which was similar to the value obtained by Rothlin[114] for cats.

Digoxin has been reported by Herrmann[91] to be fairly completely absorbed and Wayne[169] also concluded that its absorption was reasonably high. A detailed study of the absorption of digoxin carried out on 57 patients by Gold *et al.*[84] indicated that the ratio of the oral and intravenous dose was about 1.5 to 1. This evidence was in accord with that of Evans, Dick and Evans[64] who concluded that the ratio of oral to intravenous dose of digoxin was of the order of somewhat less than 2 to 1. It is evidently not as completely absorbed as is digitoxin.

The results obtained from animal experiments for k-strophanthin and ouabain appear to hold for humans as the intravenous dose of the former is 1/150-1/200 of the oral (Groedel and Kirsch[87]). Ouabain is also ineffective orally. A re-assessment of the rectal absorption of ouabain in the dog has been made recently by Raymond-Hamet[104] and it was found to be 38 times less toxic rectally than intravenously. The squill glycosides have been reported[11] to be well absorbed by the oral route but experimental figures are not available.

The ineffectiveness of some of the glycosides when used orally may be due to decomposition in the alimentary canal. This is probably true for the strophanthus glycosides which are more highly hydroxylated and hence may be more readily attacked. Reinert[116] reported that in animal experiments 60 per cent of the dose of ouabain and 35 per cent of the dose of k-strophanthin was absorbed from the duodenum if they remained there for 5 hours, indicating that low oral activity could be due to destruction by the stomach acids. These results would appear to conflict with those reported by Rothlin and Bircher[115] but the conditions of intraduodenal infusion were different.

Destruction of digitalis glycosides in the alimentary canal is also a matter for contention although the agreement obtained by so many workers using different animals on the close ratio of the oral and intravenous dose for digitoxin would indicate that this glycoside is not affected to any significant extent. One of the earlier workers in this field, Ogawa,[97] claimed that digitalis preparations were not affected by the contents of the stomach or duodenum.

Svec[148] and Brucke[illegible] on the other hand found that the gastric contents did decrease the activity of digitalis. Loss of potency has been reported in the presence of diastase, pancreatin and intestinal juice (Holste[74]) and in artificial pancreatic juice (Hale[illegible]). Friedman *et al.*[illegible] point out that although the pH of the stomach is sufficiently low to promote hydrolysis of digitoxin it is doubtful if it remains in the stomach long enough to permit any significant hydrolysis to digitoxigenin and digitoxose. Even if some did occur the impairment of activity would not be very great as the genin would be absorbed. Some of the conflicting results are no doubt due to the use of less accurate methods of biological assay by the earlier workers, and to variations in the constituents of the crude digitalis extracts or glycosides.

There is a good deal of evidence to indicate that substances present in unpurified mixtures of glycosides or in galenical preparations of digitalis modify considerably the rate of absorption. Recently Merck[illegible] reported that the activity of gitalin, a crude preparation obtained from *Digitalis purpurea* was considerably higher than that of the sum of the individually known constituents when administered intraduodenally. This preparation according to Haack, Kaiser and Spingler[illegible] contained the glycosides digitoxin, odoroside H, gitoxin, gitaloxin, strospeside and at least one other unknown glycoside. It was concluded that unknown substances which promote the absorption of the glycosides must also be present; even the poorly absorbed gitoxin series was fully absorbed from the duodenum in the presence of these constituents.

Such evidence of the difference in behaviour of glycosides when unknown plant constituents promoting their absorption are present draws attention to the uncertainty involved in using potency figures produced from intravenous experiments on animals for a crude extract, for arriving at a therapeutic dose for humans when given exclusively by mouth. Until more is known about the nature of these "absorption-promoting substances" it is impossible to predict whether or not they will be present in different plant extracts and this lends weight to the opinion frequently expressed that these preparations should be assayed after intraduodenal administration.

Chapter VI

DISTRIBUTION OF CARDIAC GLYCOSIDES IN TISSUES AND ORGANS

KNOWLEDGE of the fate of cardiac glycosides from the time they enter the blood has become more exact in recent years through the use of radioactive digitoxin, the embryonic duck heart method of quantitative analysis and paper chromatography. Most of this work has been carried out with the digitalis glycosides, mainly because of their widespread clinical use, and important differences in the behaviour of the glycosides can be attributed to variations in chemical structure.

CARRIAGE IN THE BLOOD

Digitoxin. Bine and Friedman[9] found as did Hatcher[66] that there was a rapid initial fall in blood concentration after giving rats a dose of 1.0 microgm. per gm. Only 10 per cent of the total dose was present in blood 5 minutes after injection. After this rapid initial loss the concentration fell slowly and some digitoxin could be detected in rat blood 16 hours after injection.

Using radioactive C-14 digitoxin on human subjects with cardiac failure, Okita *et al.*[101] also found an initial rapid fall in the blood level of unchanged digitoxin. Fifteen minutes after injection, about 12 per cent of the dose was present, after one hour 6 per cent, 6 hours 3 per cent, 24 hours 2 per cent and after 96 hours trace amounts to 1 per cent. A higher percentage of metabolic products of unknown constitution could however be detected. These workers attributed this rapid initial fall to the equilibration of the digitoxin with various body tissues. The biological half-life of free digitoxin in blood was estimated at 15-30 minutes. The long continued persistence of a small amount of free digitoxin in the blood was

attributed to the presence of a "loosely" bound form of digitoxin with a biological half-life of 48 to 54 hours. A third complex more strongly bound was also probably present as small amounts of material which was claimed to be digitoxin continued to be excreted in the urine 40 days after administration.

The Friedman group[47] reported a slower initial rate of clearance of digitoxin from the blood circulation of humans than was found in rats.[4] About 50 per cent of the dose disappeared from human blood within 3 minutes of injection, but the blood level remained constant at this value for one hour. After three hours no digitoxin could be detected in the blood. This rate of disappearance is also much slower than that observed by Okita *et al.*[101] for humans. However, as pointed out above Okita *et al.*[99] were able to discriminate between digitoxin and digitoxin metabolites, and found that the total carbon-14 constituents remaining in human blood one hour after injection was about 25-30 per cent of the dose. Friedman *et al.*[47] estimated the total cardioactive material in the blood and this would include metabolites of digitoxin still retaining activity but which Okita *et al.* claim to be able to separate from the digitoxin by alumina chromatography. The contrast between the results obtained in rat and human blood clearance by the Friedman group could be due to the relatively larger dose of digitoxin given to rats.

Brown, Shepheard and Wright[18] in a qualitative study (paper chromatographic), of the cardioactive constituents of rat blood and organs after digitoxin administration also found evidence of a rapid fall in blood level of digitoxin. They were unable to detect any of the glycoside or its metabolites in the circulating blood 30 minutes after an intravenous dose of 4 microgm. per gm. of body weight. Repke[113] however bulked the blood of 10 or 16 rats 13 or 24 hours after a dose of 5 or 2.5 microgm. per gm. of body weight of digitoxin, and was able to detect the presence of metabolites by paper chromatography.[114] One of these metabolites appeared to be the same as that found by Brown and Wright[15] in rat urine after high level doses of digitoxin and which was later shown by Brown, Wright and Okita[16] to be digoxin.

Lanatoside C. Friedman and Bine[44] showed that an intravenous dose of 1.2 mg. of lanatoside C disappeared from human blood

within 30 minutes. This apparent rapid rate of disappearance from the circulating blood also occurred in rats. Bine and Friedman[4] found no trace of lanatoside C in rat blood 60 minutes after an intravenous dose of 1 microgm. per gm. of body weight, although their method of analysis (embryonic duck heart) would detect the glycoside at a dilution of 1 in 20 million. Brown, Shepheard and Wright[14] examined blood of rats qualitatively for cardioactive metabolites of lanatoside C and found only traces of digoxin and *metabolite B* (p. 36) after very large doses (4 microgm. per gm. of body weight). No lanatoside C or its metabolites could be detected qualitatively in blood 45 minutes after I.V. injection

Other Digitalis Glycosides have not been studied as extensively as digitoxin or lanatoside C. The presence of digoxin and its metabolites in the blood of rats has been examined by Brown, Shepheard and Wright.[14] A few minutes after an intravenous dose of digoxin both the free glycoside and its main cardioactive metabolite *(metabolite B)* were detected in the circulating blood in approximately equal amounts. However the metabolite was cleared from the blood within 30 minutes whereas traces of digoxin were present 2-4 hours after the injection. It appears that as far as the main bulk of the glycoside is concerned, digitoxin is removed from the blood more rapidly than is lanatoside C or digoxin, but there is a good deal of evidence to indicate that only digitoxin persists in the blood and in the body in small amounts for long periods.

A qualitative study of the fate of acetyl digitoxin in rats has been made by Martin and Wright.[14] Paper chromatograms of chloroform extracts of blood after high level doses of the glycoside (4 microgm. per gm.) showed the presence of acetyl digitoxin, digitoxin and digoxin. Acetyl digitoxin appeared first in the blood but after one hour only digitoxin and digoxin were present. The digitoxin appeared to persist longer than digoxin and could be detected two hours after intraperitoneal injection.

Serum Protein Binding of Glycosides. It has been known for some time that the toxic dose of digitoxin is increased in the presence of blood serum, and this has been attributed to a binding of the glycosides with serum protein (for earlier references see Rothlin and Bircher[11]). By fractionating human, horse and rabbit serum using salt precipitation and electrodialysis methods, Lendle

and Pusch[illegible] and Haarmann *et al.*[illegible] produced evidence that digitoxin is bound only to serum albumin. These workers estimated the free glycoside colorimetrically by the relatively non-specific Baljet reaction. Rothlin and Kallenberger[illegible] investigated the binding of several glycosides with *six blood fractions (separated by electrophoresis methods by Cohn)* using the isolated frog heart method of testing. Only the fraction containing serum albumin appeared to be capable of binding digitoxin. Fibrinogen and globulin were without effect. They estimated *that 1 mg. of pure albumin* was able to bind 2.2 microgm of digitoxin. This would mean that 550 mg. of digitoxin could be found by the blood of an average man whereas the therapeutic dose is only 1-2 mg. Rothlin *et al. explain this difference in the actual and theoretical doses as being* most probably due to dissociation of the albumin-glycoside complex *when the blood expands* into the capillary system.

Rothlin (reported by Rothlin and Bircher[illegible]) has divided the glycosides tested into three groups according to their capacity to bind with serum when tested by the isolated frog heart.

Group 1. Lanatoside A, Digitoxin; require 5-10 times higher concentration in blood serum than in Ringer for a toxic dose.

Group 2. Lanatoside B, Gitoxin, Scillaren; require 3-5 times higher concentration.

Group 3. Lanatoside C, and K-Strophanthoside; equal effect in blood serum and Ringer solution.

A similar gradation was found with serum albumin, and Rothlin[illegible] *attributed the difference in albumin binding* amongst the glycosides tested to the presence and position of hydroxyl groups in the steroid aglycone and not to the type or number of sugar residues present in the molecule *The most strongly bound have only* two hydroxyl groups in their aglycone (digitoxigenin at C_3 and C_{14}). Those only weakly bound or not bound at all have three hydroxyl groups *in their aglycones (digoxigenin* C_3, C_{12} C_{14}, and strophanthidin C_3, C_5, C_{14}). An intermediate group is derived from *gitoxigenin which has 3 hydroxyl groups* at C_3, C_{14}, and C_{16} (with the C_{16} hydroxyl probably hindered by the unsaturated lactone ring).

The rapidity of action of the glycosides *(latent period) has been* found by animal experiments and clinical trials to parallel this ap-

parent order of binding with serum albumin. Lanatoside A and digitoxin act much more slowly than lanatoside C or k-strophanthin when injected intravenously. Thus, in cardiac insufficiency the heart output increases within 10 minutes after lanatoside C is injected intravenously (Largerlof and Werko reported by Rothlin and Bircher[illegible]) and a full therapeutic effect is reached within 1-1 1/2 hours. Digitoxin which is a "bound" glycoside requires 4-8 hours for its effect to reach a maximum (de Graaf[illegible]).

It would appear that the serum proteins do not bind all the digitoxin from solution. Farah[illegible] found no difference between the lethal dose obtained with digitoxin bound to serum albumin when tested on the intact cat and dog and the heart lung preparation. Friedman and co-workers[illegible] found that 1 ml. of human serum containing 0.4 microgm. of digitoxin still exerted cardioactive properties although the amount of albumin present was sufficient to bind 50 microgm. of digitoxin. Evidently an equilibrium exists between the protein and the glycoside.

The question of blood protein binding of digitoxin has recently been re-investigated by Spratt and Okita[illegible] using radioactive digitoxin. Blood protein—digitoxin C-14 mixtures prepared by *in vitro* incubation of human serum and by separation of rat blood after an intravenous injection of the radioactive glycoside, were separated by electrophoresis on starch beds and also by the salt fractionation—dialysis method used by earlier workers. No evidence of binding of digitoxin by serum albumin was obtained in the electrophoretic separation of either *in vitro* or *in vivo* serum-digitoxin preparations. Evidence of an increase in digitoxin—albumin "binding" was observed as the albumin was purified by salt fractionation methods similar to those used by earlier workers However, these protein fractions obtained by this method were shown by electrophoretic studies to be somewhat heterogeneous. Spratt and Okita also estimated the "cholesterol" and lipid phosphorus content of the serum protein fractions obtained by salt precipitation, and the results pointed to a positive correlation between the digitoxin content of the protein fractions and their possible lipo-protein content. As this work conflicts with previous efforts to relate the binding effect of serum to the albumin fractions a re-evaluation of the position is needed. The possible role of lipo-

proteins or blood lipids needs further examination. The fact that the degree of binding of the glycosides with serum increases as the number of hydroxyl groups of the aglycones decreases, may be due to an increase in lipid solubility which is also observed when the polar properties of a molecule are decreased.

DEPOSITION IN THE TISSUES AND ORGANS (DIGITOXIN)

The use of radioactive tracers and of more sensitive biological methods of analysis has also enabled direct estimation of the cardioactive material present in organs and tissues after the administration of cardiac glycosides. Most of this work has been carried out with digitoxin. The widely held view that the heart must contain a higher concentration of the drug than other organs has now been proved to be erroneous. Doubt had been cast upon this idea by Hatcher and Eggleston,[illegible] and the more recent work of *Dock, Stockton, and Wood,*[illegible] *Farah,*[illegible] *Farah and Smuskowicz*[illegible] emphasized the greater importance of the liver in binding glycosides.

Bine *et al.*[illegible] using the embryonic duck heart method of assay found that the immediate deposition of digitoxin was greatest in the liver of the rat, and that the amount bound by the heart per gram of tissue was approximately one third that bound by the liver. These workers also showed that the heart did not retain digitoxin longer than other organs. The role of the liver of the rat in excretion of digitoxin was found to be relatively unimportant (St. George, Bine and Friedman[illegible]) as only 10 per cent of the dose was eliminated through the bile. Digitoxin was, however, found to disappear from the liver 12 hours after injection so that this organ must destroy or change the glycoside (Friedman, St George, Bine, Byers and Bland.[illegible]).

Comparison of the behavior of digitoxin in rats, rabbits and dogs (Friedman *et al.*[illegible]) showed that in all three animals, the heart had no special affinity for the glycoside, the organs of excretion containing more cardioactive material per gram of tissue. Some difference was found in the distribution of digitoxin in these animals. The kidney of the dog for example contained a higher proportion of the drug than the kidneys of rats or rabbits, although very little digitoxin was actually excreted in the urine of any of these animals.

Experiments with radioactive digitoxin by Fischer, Sjoerdsma and Johnson[illegible] gave a similar picture and the advantage of the use of "normal" doses make the results of more direct application. Also, for the first time in studies of tissue distribution of cardiac glycosides a distinction was made between unchanged glycoside and its metabolites, although it was not possible to indicate whether or not these metabolites were still cardioactive. Again the liver of the rat and cat played a major part in the metabolism of the glycoside, but it appeared that the glycoside and its metabolites persisted in the heart of the cat whereas little or no fixation was found in the rat heart. This may help to explain the well-known insensitivity of the rat towards cardiac glycosides when compared with other experimental animals. Another interesting fact revealed by this work was that the kidney of the cat maintained a high concentration of digitoxin whereas little or none was found in the rat kidney. As cat urine contained very little digitoxin, but a high concentration of metabolites, Fischer *et al.*[illegible] considered that the cat (and perhaps other sensitive animals) selectively excreted metabolites and retained the unchanged glycoside.

A direct comparison of the uptake of radioactive digitoxin by isolated rat, guinea pig, rabbit and cat hearts was made by Sjoerdsma and Fischer[illegible] The more rapid uptake of the glycoside by the heart which occurred in the earlier stages of perfusion (a fact observed by Straub[144]) was thought to be due to the receptive capacity of the heart tissue for the glycoside becoming exhausted after one passage of digitoxin through it. Sjoerdsma and Fischer[illegible] observed a difference in the amount of glycoside bound by the different animals. The rat and guinea pig hearts bound more than twice as much glycoside per gram of tissue than rabbit and cat hearts, an observation which conflicted with the *in vivo* work of Fischer, Sjoerdsma and Johnson,[illegible] but the period of infusion differed in different experiments (15-20 minutes for the rat and guinea pig hearts and 5-10 minutes for the rabbit and cat hearts).

Okita *et al.*[illegible] have been able to give a picture of the distribution of radioactive digitoxin in humans (see also Okita[illegible]). Three patients were given doses of radioactive digitoxin shortly before death and the relative concentrations of the glycoside and its metabolites in the organs and tissues were estimated after autopsy.

The total amount of unchanged digitoxin was highest in the liver, the myocardium showing no selectivity. The cardiac tissue did however show a higher concentration than skeletal muscle. This could be due to the glycoside being more available to the heart than to skeletal muscle by carriage in the blood stream immediately after injection. The amount of unchanged digitoxin in the ventricle was as low as 2 microgm. per 100 g. of tissue, indicating how little of the glycoside is necessary for cardiotonic action.

On a tissue weight basis the highest concentration of unchanged digitoxin was found in the colonic contents, and this was followed by the gall bladder contents, jejunal contents, kidney, ileal contents, lung, liver, jejunum, ventricle and auricle. The concentration in blood was very low as indicated previously (p. 45). In all organs studied there was a higher concentration of metabolic products than unchanged glycoside, and the metabolite:digitoxin ratio indicated that the liver was apparently the most important centre for the chemical change of the glycoside. The values obtained for the relative concentrations of unchanged glycoside and metabolites in the various organs have led Okita *et al.*[111] to postulate an entero-hepatic cycle for digitoxin and its metabolites. The significance of this hypothesis will be discussed later (Section IX).

The presence of cardioactive metabolites in the tissues of rats after digitoxin administration was first reported by Brown, Shepheard and Wright.[10] It was found that 15 minutes after an intraperitoneal injection of high doses of digitoxin, the unchanged glycoside and a metabolite of it were present in liver in approximately equal quantities as judged from color intensities on paper chromatograms. Digitoxin but not its metabolite was detected in kidney but very little cardioactive material (as indicated by the presence of molecules giving the Raymond reaction) could be detected in other organs because of the relative insensitivity of the methods used. Repke[111] however, by using a large number of rats and bulking the tissues together was able to detect metabolites of digitoxin in blood and most organs 12 or 14 hours after an intravenous injection of 5.0 microgm. or 2.5 microgm. of digitoxin per gram The chief metabolite appeared to be digoxin which had previously been found only in the urine (Brown *et al.*[9]) or in bile (Cox and Wright[25]).

Repke[11] also determined the digitoxin content of the tissues and organs of rats after high doses of digitoxin (5 microgm. per gm.) by colorimetric estimation with xanthydrol after concentrating the cardioactive materials on alumina columns. The metabolites of digitoxin could not be separated from the parent glycoside by this method and no xanthydrol color reaction would be given by metabolites lacking digitoxose residues (e g., digitoxin *metabolite C*, p. 34). Repke's results however again indicated the importance of the liver as a centre of metabolism of the glycoside, but the adrenal glands showed comparatively high cumulative ability. The heart only bound 0.1% of the dose (compared with 23 per cent for the liver) and this finding further confirmed the modern view that heart muscle has no special affinity for digitoxin.

Other Glycosides. The distribution of other cardiac glycosides in animals has not been studied very extensively as radioactive glycosides other than digitoxin have not been available. Brown, Shepheard and Wright[10] found significant qualitative differences in the behavior of digoxin, digitoxin and lanatoside C in rats. The importance of the liver was again made obvious by this work as the rate of blood clearance seemed to be dependent upon the binding capacity of the liver more than by any other factor. Metabolites of all three glycosides were detected in the liver immediately after injection, digitoxin apparently remaining longer in the liver than did the other glycosides but the metabolite of digitoxin disappeared more rapidly from the liver than did the metabolites of lanatoside C and digoxin.

The chief metabolite of digoxin in liver was the same as found in urine after digoxin administration (see Section VII) and the nature of this metabolite *(metabolite B)* has been discussed in Section IV) Apparently the digitoxose residues are removed very quickly after the glycoside is injected and an unknown conjugate replaces them. This metabolite could also be detected in heart, kidney and blood. Lanatoside C was apparently split in the liver to digoxin and to *metabolite B* and traces of digoxin and *metabolite B* could be detected in blood, heart and kidney. No free genins of these glycosides were detected in any organ or tissue, a fact which was also noted by Repke in the case of digitoxin.

After acetyl digitoxin administration the livers and hearts of rats

showed the same substances found in blood after dosage with the glycoside, *viz.*, acetyl digitoxin, digitoxin and digoxin.[illegible] In the kidney however, in addition to these substances, digoxin *metabolite B* was present. This substance also appears in urine (see Section VII) and appears to be an excretory substance mobilised by the kidney and excreted in urine.

Little exact information is available about the distribution of squill and strophanthus glycosides. It is difficult to assess the earlier work because of the inexactness of the methods of analysis used. Recent work of Giertz, Hahn and Schunk[illegible] investigated differences in the accumulation of several glycosides in the liver and kidney of guinea pigs. The method used for measuring partition was similar to that of Rothlin — eliminating the organ and then determining the lethal dose by the method of Hatcher and Brody.[illegible] It was found that the binding of cardiac glycosides to the liver was in the following order: digitoxin, scillaren A, k-strophanthin, and the binding to the kidney in the order, k-strophanthin, scillaren A and digitoxin. The order of binding of these glycosides in the liver followed that observed by Rothlin[illegible] for serum albumin binding and Giertz *et al.*[illegible] suggested that this may be due to the liver being the site of formation and storage of albumin. No doubt other correlating factors such as solubility in liver lipids could also be found, and this explanation could mean very little.

SUB-CELLULAR DISTRIBUTION OF CARDIAC GLYCOSIDES

[illegible] made to determine whether *digitoxin* [illegible] view [illegible] ction of cardiac glycosides. St. George, Friedman and Ishida[illegible] using embryonic duck hearts analyzed the cardioactive material present in different fractions of the centrifugates of homogenized rat liver and hearts after intravenous injection of digitoxin. They found that digitoxin was apparently capable of entering the cell and that more than 85% of the cardioactive material was present in the supernatant fraction of both heart and liver, while mitochondria contained only small amounts (3% in heart and 0.1% in liver). This suggests that digitoxin does not exert its effect upon the

energy-producing systems, thus supporting other evidence (Wollenberger[144]).

Similar results were obtained by Spratt and Okita[138] using *in vivo* and *in vitro* (liver slice) methods to study the cellular distribution of C-14 and H-3 labeled digitoxin. In this work a distinction was made between unchanged digitoxin and its metabolites as evidence of rapid metabolism of the glycosides was obtained Over 90% of the unchanged digitoxin was found in the soluble supernate of fractions of rat heart, liver and kidney cells. There was no difference between *in vivo* and *in vitro* studies. These results differed from those of Harvey and Pieper[61] who found that 43% of the digitoxin was present in the particulate fraction and 57% in the soluble supernate of homogenates of guinea-pig hearts after perfusion with C-14 digitoxin. No attempt was made to separate the digitoxin from its metabolites in this work but a species difference cannot be excluded. Perhaps as St. George *et al.*[134] state, "the accumulation of digitoxin in guinea-pig mitochondria provides a basis for the extreme sensitivity of the guinea-pig to glycosides as opposed to the notorious insensitivity of the rat." It would appear that further examination of this problem is needed.

Chapter VII

THE EXCRETION OF CARDIAC GLYCOSIDES

THE FINAL FATE of cardiac glycosides in the animal, their excretion through the kidney, bile and intestine, has only been accurately assessed in recent years through the use of the sensitive analytical methods previously described. There is little to be gained in a detailed review of the earlier work because most of this has had to be repeated and only those results of historic interest need be mentioned.

Renal Excretion. The strophanthus glycosides appear to have been the first glycosides used in urinary excretion experiments. In 1909, Hatcher[illegible] found that ouabain was excreted in very small amounts in rat urine, and a more intensive study of the excretion of ouabain, strophanthidin and digitoxin in the rat and the cat by Hatcher and Eggleston[illegible] confirmed that very little of these glycosides were excreted after oral administration. Quantitative studies were made in later years on various laboratory animals (Santesson and Ekstrom,[illegible] Fischer[illegible] and Lendle[illegible]) but all of these workers were hindered by the lack of sufficiently sensitive biological or chemical methods of estimation. Furthermore, because of the lack of precise knowledge of the chemical nature of the glycosides, and of methods of separating small amounts of cardioactive materials no attempts were made to distinguish between the glycosides and their metabolic products.

Digitoxin. The renal excretion of digitoxin has been reviewed by Friedman, St. George and Bine.[illegible] Relatively small amounts of cardioactive material are excreted in the urine of laboratory animals after digitoxin administration, but estimations based on embryonic duck and chick hearts are all lower than those obtained *by the use of radioactive glycosides. This may* be due to the use of smaller doses (in the "therapeutic range") of cardiac glycosides,

since the large doses required to give assayable quantities in urine when biological assays are employed result in a diminishing return of active material excreted and hence small percentage recoveries are obtained. The radioactive studies also permit a distinction to be made between original glycosides and metabolites, but it is not possible to determine whether the "metabolites" of the radioactive glycosides are cardioactive because of the small amounts involved. The separation of closely related metabolites from the original glycosides on alumina columns as used by Okita *et al.*" and Fischer *et al.*" is a difficult operation, particularly when small quantities are involved, and analytical figures based on such separations should not be interpreted too precisely.

The results of radioactive studies in laboratory animals by Fischer *et al.*" indicated that in rat urine 8 per cent of the dose was excreted as unchanged glycoside during the first 48 hours. Radioactive metabolites accounted for a further 20 per cent of the dose. Cats excreted about 3 per cent of the unchanged glycoside in urine over a period of 7 days although 64 per cent of "metabolised" glycoside was excreted during the same period.

The biological assays (embryonic duck heart) of Friedman *et al*" showed only 3 per cent of cardioactive material in rat urine after a large dose of 1 microgm. per gram. This excretion occurred during the first 24 hours; no glycoside could be detected on subsequent days. Brown, Ranger and Wright' obtained a similar figure for rats using embryonic chick hearts for assay, but showed by paper chromatography that the urine contained very little unchanged glycoside, most of the cardioactive material consisted of a conjugate of digoxigenin (see p. 35). This metabolite would most probably be less active biologically on chick or duck embryo hearts (digoxin is less active than digitoxin by a factor of about 3.4 on embryonic chick hearts) and this may explain the comparatively slight difference between the biological and radioactive determinations. It should be noted however that cardioactivity of the chloroform extract of urine as assessed by the embryonic chick heart before paper chromatography, was approximately the same as that of the sum of the metabolite and digitoxin after separation on paper chromatograms This indicated that there was no significant amount of cardioactive substances present in the

urine which did not react with m-dinitrobenzene and hence would not contain the unsaturated lactone ring.

Repke[112] estimated the total "digitoxin" in rat urine by a colorimetric method after alumina column chromatography of chloroform extracts as 2.5 per cent of the dose (5 microgm. per gm.) after 12 hours. This author stated that the urine contained a metabolite more polar than digitoxin, and in another communication[113] showed that this metabolite was probably digoxin by the use of paper chromatography. The metabolite was present in greater quantity than digitoxin.

Further results of St. George *et al.*[111] showed that the urinary excretion of cardioactive material in dogs and rabbits after digitoxin administration is very low. Apparently the only laboratory animal which excretes any significant amount of cardioactive material is the frog. This was reported by Lendle[89] to excrete 40-50 per cent of the injected dose of digitoxin.

The excretion of digitoxin by humans has also been studied extensively with normal and radioactive glycosides. Friedman, Bine, Byers and Bland[48] found that unlike laboratory animals a good deal of cardioactive material was excreted in urine following doses of digitoxin, but that it was spread over a long period. Young adults were estimated to excrete 40-50 per cent of a single dose over two to three weeks. From 4 to 5 per cent was excreted in the first 24 hours and the amount excreted per day gradually fell over the three week period. With elderly subjects the first day excretion appeared to be only about one per cent, but the total excretion was not estimated. However, young normal adults on a maintenance dose of 0.1 mg. daily excreted about 30-40 per cent of the dose each day, and this apparently represented complete "digitalization." When the dose was increased signs of toxicity appeared and the urine content of cardioactive material almost doubled. Friedman *et al.*[48] conclude from this evidence that "normal man and the cardiac patient cannot rid themselves of more than about 50 microgm. of digitoxin per day by extra-renal processes, irrespective of the amount given. This fact, of course, explains the ease with which toxicity is obtained following use of this steroid."

With cardiac patients some differences in excretion were noted by Friedman *et al.*[48] Patients with acute left ventricular failure

excreted digitoxin as readily as normal subjects, but patients suffering from right ventricular failure showed low levels of excretion during the first 24 hours, but reached normal levels during the second and third days after the dose as the state of congestive failure and renal excretion improved. Patients on maintenance doses of digitoxin exhibited a similar excretory pattern to normal subjects.

These estimations of Friedman and co-workers assumed that only digitoxin was present in human urine. Although no quantitative estimations were carried out, Ashley, Brown, Okita and Wright[illegible] demonstrated that human urine contained digoxin, digoxin *metabolite B* (=*metabolite C*) as well as digitoxin. The original glycoside appeared to be present in lower concentration than its metabolites.

Differences in the biological activity of the digitoxin metabolites may partly explain the different results obtained from the use of radioactive digitoxin in a study of its urinary excretion by humans. Okita *et al.*[illegible] reported that 60 to 80 per cent of a dose of C-14 digitoxin administered to patients with cardiac insufficiency was excreted via the kidney. Of this however, about 60-80 per cent consisted of metabolites and only 6-10 per cent was the parent glycoside. A marked excretion (about 20 per cent) occurred during the first three days after dosage, but minute amounts of unchanged digitoxin were detected in the urine up to forty days after the dose, and metabolites up to eighty days. The long excretion of radioactive metabolic products is probably not significant, but the rate of excretion of unchanged glycoside indicated that digitoxin has a half-life period of approximately nine days. This figure agrees well with the clinical evidence for the cumulative action of digitoxin.

The differences in excretory rates as estimated by Friedman and co-workers[illegible] and Okita *et al.*[illegible] need to be reconciled by further work. The fact that the radioactive studies show a greater excretion of metabolites than glycoside is not surprising when the work on the metabolites of digitoxin in rat urine is considered. Undoubtedly the metabolites include digoxin and *metabolite B*, and hence will have biological activity. Both active metabolites and parent glycoside would therefore be included in the excretory figure arrived at by Friedman and co-workers and this helps to reduce the

differences observed, provided it is assumed that a clear separation of their substances was obtained in the extraction and purification process used in the radioactive work. It is more difficult to reconcile the rapid initial excretion found after administration of radioactive glycoside with the low daily recoveries obtained by biological estimation.

OTHER DIGITALIS GLYCOSIDES

Other digitalis glycosides have not been as exhaustively studied as digitoxin. Quantitative figures for the urinary excretion of digoxin, acetyl digitoxin and lanatoside C by rats are available however and a comparison of the effect of slight molecular structural differences is of interest.

The urinary excretion of lanatoside C by rats was estimated by Bine, St. George and Friedman[7b] at 17 per cent for the first 24 hours; no significant amount was excreted on subsequent days. This higher rate of excretion compared with digitoxin was attributed by these workers to the lower binding capacity for serum albumin claimed by Rothlin and Kallenberger[100] and its rapid disappearance from blood. Brown, Ranger and Wright[8] arrived at a lower figure of 7.5% (6.8-8.3) for the first 24 hours urinary excretion by rats at the same dose level (1 microgm. per gm.) as used by the Friedman group. The urine contained lanatoside C only at low dose levels but at higher levels digoxin and digoxin *metabolite B* could be detected well.

The digoxin excreted in rat urine was also estimated by Wright and co-workers[10] using the embryonic chick heart together with paper chromatography. With doses of 1 microgm. per gm. the total cardioactivity of the first 24 hours urine was equivalent to 9.6 per cent of the dose. Of this about 6 per cent was unchanged digoxin and 4 per cent digoxin *metabolite B*. The total excretion would be slightly higher if the biological activity of the digoxin metabolite is less than digoxin on the embryonic chick heart.

Acetyl digitoxin is excreted in rat urine at only a slightly higher level than is digitoxin. Martin and Wright[11] using colorimetric methods, estimated the cardioactive substances present as approximately 4-6% of the dose. The urine contained acetyl digitoxin and digitoxin in very small amounts, the chief excretory products

being digoxin and digoxin *metabolite B* present in proportions of approximately 1 to 4.

It is apparent that as far as the rat is concerned, very little of the dose of a cardiac glycoside appears in urine in the form of the original glycoside or its cardioactive metabolites. The use of radioactive glycosides could show to what extent non-cardioactive metabolites are present in the urine. The well known resistance of the rat to cardiac glycosides may be due to the capacity of the tissues and organs of this animal to metabolize them more intensively to produce non-cardioactive molecules. The differences in the fate of the digitalis glycosides in rats are summarized in Table V.

TABLE V

THE URINARY EXCRETION OF CARDIAC GLYCOSIDES BY RATS

Excretion in first 24 hours, dose level 1 microgm per g

Glycoside	*Mean Per Cent Dose Recovered*	*Limit of Error P = 0.95*	*Form Excreted*	*Reference*
Digitoxin	3	-	—	45
Digitoxin	2 2	19 8 - 2 5	digitoxin + *metabolite B*	9
Acetyl digitoxin	5 4	3 8 - 7 0	*metabolite B* digoxin acetyl digitoxin digitoxin	94
Digoxin	9 6	7 5 - 11 5	*digoxin* metabolite B	130
Lanatoside C	7 5	6 8 - 8 3	lanatoside C	9
Lanatoside C	17 0	-	—	5b

(The predominant excretory product is in italics)

No quantitative figures are available for the urinary excretion of these glycosides by humans. Ashley, Brown, Okita and Wright[9] reported that the same metabolites are found in human urine as in rats after 1 milligram doses of lanatoside C and digoxin. Thus digoxin gave *metabolite B* and some unchanged digoxin, while lanatoside C yielded digoxin, digoxin *metabolite B* and unchanged lanatoside C. The relative proportions of the metabolites present in the urine were not estimated.

THE HEPATIC EXCRETION OF CARDIAC GLYCOSIDES

Digitoxin. Most of the detailed work on the biliary excretion of cardiac glycosides has been done with digitoxin, and as with

urinary studies, information has been gained only in recent years.

St. George *et al.*[134] studied the biliary excretion of digitoxin in several laboratory animals. *Rats were estimated to excrete only* 10 per cent of the dose (calculated as digitoxin) in the first 24 hours. Rabbits and dogs also excreted digitoxin in bile at about the same level as did rats so that this route does not appear to account for a major portion of the dose. Geiling *et al.*[60b] using *radioactive digitoxin also estimated that about 11* per cent of the total radioactivity administered appeared as parent glycoside in the bile of dogs collected for 48 hours after a dose.

Cox and Wright[90] also found that the rat excreted 8-12 per cent of the dose in the bile. This amount was however excreted within the first 5 hours after dosing. The bile contained not only the parent glycoside (5.0-7.0 per cent) but also 3.0 to 5.0 per cent of digoxin indicating rapid hydroxylation at position 12 in the steroid nucleus of digitoxin.

Excretion of digitoxin in the bile of humans with biliary fistulas was followed by St. George *et al.*[134] but they were unable to detect any cardioactive material using the embryonic duck heart method. This would indicate that less than 0.5 microgm. of digitoxin per ml. of bile was excreted, amounting to less than 20 per cent of the dose. Okita *et al.*[141] were able to arrive at a more accurate figure for biliary excretion after giving radioactive digitoxin to humans and examining the bile post mortem. Only 1 to 2 per cent of the administered dose was present in the bile sample taken 36 hours after administration, but digitoxin metabolites amounting to 10-17 per cent of the dose were present. The nature of these metabolites was not determined and the significance of these results in relation to the possible circulation of digitoxin and its metabolites in humans will be discussed later.

Other Glycosides. The polarity of individual glycosides appears to have an important influence upon their biliary excretion. The polarity of a molecule is often an indication of its relative solubility in water and lipids, and in the cardiac glycosides the number and position of the hydroxyl groups present in the molecule as well as the type of sugars present have an important influence on their physical properties (Schindler and Reichstein,[133] Heftmann and Levant,[64] Harrison and Wright[84]). Ouabain is one of the most

water-soluble glycosides and it has 5 free hydroxyl groups in its aglycone. Farah[illegible] found that in rats it was excreted in the bile to the extent of 80-85 per cent of the dose, 2-4 hours after intravenous administration. On the other hand, only 8-12 per cent of the dose of the relatively non-polar glycoside digitoxin (one free hydroxyl group in the aglycone) is excreted in rat bile.[illegible]

A study of digitalis glycosides by Cox and Wright[illegible] and by Martin and Wright[illegible] illustrates the influence of molecular structure on excretion in rat bile. The glycosides lanatoside C, lanatoside A, digoxin, digitoxin and acetyl digitoxin show a progressive decrease in polarity (as revealed by their Rf values on different systems of paper chromatography) and the amount excreted in 5 hours falls from 60-80 per cent for the lanatosides to 7-10 per cent for acetyl digitoxin. Furthermore, the glycosides which are excreted in greater amounts show more tendency to be excreted unchanged than as cardioactive metabolites (see Table VI).

The correlation of polarity with rate of excretion could be interpreted as being due to the relative water solubilities of the glycosides. There is however little difference in water solubility between digoxin and digitoxin. The difference in excretion could be due to differences in solubility in tissue lipids as those glycosides possessing polar aglycones or polar sugar residues (such as glucose in the lanatosides) would almost certainly be less soluble in lipids present in liver and other organs or tissues The more lipid soluble glycosides would therefore tend to remain longer and hence be more completely metabolized

INTESTINAL EXCRETION

Whether cardiac glycosides are excreted through the wall of the gut itself appears to be a controversial question. Hatcher and Eggleston[illegible] claimed to find about 10 per cent of a dose of digitoxin in the feces of rats, and this was not altered when the bile duct was ligated On the other hand St. George and Friedman[illegible] showed fairly conclusively that the whole of the cardioactivity of the feces of rats after dosing with digitoxin was due to biliary excretion Where rats with biliary fistulas were used no cardioactive substances were present in their feces, whereas about 6 per cent of the glycoside (the amount found in the feces of normal rats after 24

TABLE VI

THE INFLUENCE OF CHEMICAL STRUCTURE ON THE BILIARY EXCRETION OF CARDIAC GLYCOSIDES IN RATS

Glycoside	Number of Free Hydroxyl Groups in Aglycone	Sugars Present	Total Amount Excreted (Per Cent of Dose)	Form Excreted	References
Ouabain	5	Rhamnose	80-85	unknown	37
Lanatoside C	2	3 digitoxose + glucose	60-80	unchanged glycoside	30
Lanatoside A	1	3 digitoxose + glucose	60-80	unchanged glycoside	30
Digoxin	2	3 digitoxose	33-47	unchanged glycoside 10% metabolite B 30%	30
Digitoxin	1	3 digitoxose	8-12	unchanged glycoside 5-7% digoxin 3-5%	30
Acetyl digitoxin	1	3 digitoxose	7-10	unchanged glycoside digitoxin digoxin metabolite B.	94

hours) could be recovered from the first 24 hours biliary excretion. These results would also indicate that none of the digitoxin excreted in the bile was reabsorbed from the intestine.

Geiling *et al.*[46] however reported a species difference in that 10 per cent of a dose of radioactive digitoxin could be recovered as unchanged digitoxin from the feces of dogs with biliary fistulas.

Fischer *et al*[44] found that rats excreted 72% of a dose of radioactive digitoxin in feces and of this 25% appeared to be unchanged glycoside. This is almost four times the amount found by St George and Friedman[111] in feces and by Cox and Wright[30] in rat bile. Fischer *et al.* also found that the cat excreted 11 per cent of the dose as unchanged glycoside and 33 per cent in the form of "metabolites." This work did not indicate whether the whole of the fecal excretion was due to biliary excretion or partly to excretion by the gut.

Using colorimetric methods of assay Repke[101] estimated that the feces of rats 12 hours after a large dose of digitoxin (5 microgm.

per g.) contained only 0.9 per cent of the dose. The same author[113] was able to identify digoxin in the large and small intestine (including contents) and also in the feces of rats after this high dose of digitoxin.

No studies have been made of the excretion of cardiac glycosides by humans with biliary fistulas, but Okita *et al.*[108] were able to estimate the fecal excretion of radioactive digitoxin in two humans. Approximately 9 per cent of the original dose was excreted as unchanged digitoxin and 8 per cent as metabolic products. The amount of unchanged glycoside in the feces was approximately the same as found in the urine of these patients although 60-80 per cent of the administered dose was ultimately excreted in the urine in the form of unchanged glycoside and metabolites of it.

formation of *metabolite B* and it is not surprising to find the genin in incubation experiments.

Lanatoside C. This glycoside yielded *metabolite B*, digoxin and traces of a metabolite suspected of being digoxigenin after incubation with the following:— rat liver slices, rat kidney slices, rat heart slices and rat blood (Figure 14 ii).

Digitoxin, unlike lanatoside C and digoxin, appeared to yield much smaller amounts of metabolites which were often difficult to detect. This is in accord with the slow excretion of digitoxin and its metabolites observed in urine and in bile[4,5,6] and may indicate that the glycoside is not as readily available for metabolic attack because of immobilization in the tissue homogenates or slices, just as it appears to be in the intact animal. Metabolites which do not give the Raymond reaction could also be present, but so far it has not been possible to detect such substances.

Figure 14. Paper chromatograms of extracts from tissue slice and tissue homogenate incubations of cardiac glycosides. Formamide impregnated paper. Developing solvent— chloroform, benzene/formamide. (i) Digoxin Incubation. (ii) Lanatoside C Incubation. (iii) Digitoxin Incubation. I = digoxigenin; B = *metabolite B* (= *metabolite C*); D = *digoxin*; F = *metabolite F*; L = lanatoside C; Dg = digitoxin.

[illegible] after [illegible] with [illegible] i) no metabolites could be detected in rat heart or kidney slice incubations carried out under the same conditions as for the other glycosides. The formation of *metabolite B* and digoxin under these

conditions indicates the presence of enzymes capable of 12-β hydroxylation of the cardiac aglycone in liver and in blood. The ability of blood dilutions to promote 12-β hydroxylation is interesting because the enzymes associated with them is usually regarded as being necessary for the oxidation reaction (see for example Brodie,[7] Brodie, Gillette and La Du[6] and Grant[11]). Further experiments are needed to determine whether the apparent inability of kidney and heart tissue to hydroxylate digitoxin is real or due to experimental factors not yet determined.

Isolated Whole Organ Perfusates

The metabolism of digoxin, digitoxin and lanatoside C has also been studied by means of organ perfusates[4]. The glycosides, dissolved in Ringer-Locke solution aerated with carbogen, were perfused through isolated rat livers, kidneys and hearts for periods of 90 minutes at 37 deg. The glycoside concentration was 3 mg. in 60 to 80 ml. of circulating solution. The perfusate solutions were extracted with chloroform and the extracts examined qualitatively by paper chromatography. The following metabolites were detected in perfusates of rat livers.

DIGOXIN ⟶ *metabolite B* + *metabolite F*
LANTOSIDE C ⟶ *metabolite B* + *digoxin*
DIGITOXIN ⟶ *metabolite B* (= *metabolite C*) + digoxin.

[illegible] with digitoxin also contained traces of [illegible] perfusates. The perfusion rate in [illegible] 0.5 ml. per minute) was however very slow compared with [illegible] perfusates (about 20 ml. per minute). This would result in only two complete passages of the perfusion fluid through kidneys and heart compared with about 30 for liver. These different rates might account for the low yields or absence of metabolites obtained in kidney and heart perfusions

[illegible] of Cardiac Glycosides in Human Blood

[illegible] lanatoside C and [illegible] in the formation [illegible] been shown[4] that

human urine contained the same metabolites of these glycosides as did rat urine after suitable doses, and it might be expected that human blood would also produce the same chemical transformations under the same conditions as rat blood. Experiments designed to test this possibility have been carried out in this laboratory.[11]

Human blood diluted with an equal volume of Ringer-Locke and aerated with carbogen was incubated at 37°C for two hours with digoxin, lanatoside C and digitoxin (20 to 50 mgm. per 300 ml. of fluid). The glycosides and metabolic products were separated by coagulating the blood by boiling for 3 minutes, centrifuging, removing fat with carbon tetrachloride and then extracting with chloroform. The chloroform extracts were then used for paper chromatography. Digitoxin was also incubated under the same conditions with human plasma and serum. The following transformations were recorded.

DIGOXIN	—whole blood→	*metabolite B*, *metabolite F* and traces of digoxigenin
LANATOSIDE C	—whole blood→	*metabolite B*, digoxin
DIGITOXIN	—whole blood→ (plasma, serum)	*metabolite B*, (*metabolite C*) digoxin + digoxigenin

Control experiments in which the glycosides were incubated under the same conditions but without the blood products showed no evidence of chemical transformation.

It must be concluded that human blood (serum or plasma) contains enzymes capable of (a) hydroxylating the steroid aglycones of digitoxin (b) hydrolysing the glycosides at the glucose and digitoxose linkages and (c) conjugating the C-3 hydroxyl group of the aglycone. Whether these enzyme systems are the same as those present in cellular microsomes (liver microsomes in particular) requiring reduced triphosphopyridine nucleotide is unknown. The origin of these enzyme systems is also a matter for speculation and it would be interesting, if possible, to compare the *in vitro* metabolic activities of blood and of liver. Although there is a good deal of evidence from pharmacological experiments (see for example Giertz *et al.*[11]) that the liver is a major factor in the elimination of cardiac glycosides, no consideration appears to have been given to the possibility of blood playing an active metabolic role either in regard to cardiac glycosides or any other foreign molecule.

DISCUSSION

THE INTRODUCTION of new tools for the analysis of cardiac glycosides in biological materials in the past few years has enabled a more exact assessment of some of their pharmacological properties. One of the most important facts which has emerged is the lack of any specific affinity of cardiac muscle towards the glycosides The view previously widely accepted was that these substances probably affected all types of muscular contraction, but because of a selective ability possessed by cardiac muscle the effect was mainly observed in the heart. It would appear that extremely small amounts of the original glycosides or metabolites still possessing their important pharmacodynamic groups are able to stimulate the force of contraction of heart muscle in a manner as yet unknown. Although the indications are that only those metabolites which still possess the unsaturated lactone ring are cardioactive, the possibility exists that the glycosides may be converted to molecules of unknown type which cannot be detected by the normal biological or chemical testing methods. A more intense study of the metabolites using radioactive glycosides should yield profitable results.

Some of the well-known differences in the pharmacological behaviour of the cardiac glycosides used clinically can now apparently be attributed to the way they are metabolized or excreted by the animal. The following facts have emerged, mainly from comparative studies on rats.

1. The glycosides which are of the more polar type, due to the presence of polar sugar molecules in the glycoside or extra hydroxyl or carbonyl groups in the steroid aglycone, are more rapidly excreted either in urine, or more particularly, in bile. Digitoxin, the most cumulative glycoside in clinical use is the least polar of those studied, and is excreted in small amounts only partly as unchanged glycoside, but mainly in the form of its cardioactive metabolites in bile and in urine.

2. The more polar molecules tend to be excreted in the form of

unchanged glycosides, whereas those which remain longer in the body are more likely to be converted to metabolites.

3. *The main metabolic pathway of digitoxin (in humans as well as in rats)* involves hydroxylation and conjugation. These reactions tend to render the molecule more polar and hence reduce solubility in lipid tissue. The lipid solubility of the glycosides appears to be an important property in relation to absorption, metabolism and excretion. An interesting feature emerging from the study of the metabolites of the digitalis glycosides is the presence of the substance known as *metabolite B* — an unknown conjugate of digoxigenin — in almost all organs and excretory products after the administration of digitoxin, acetyl digitoxin, lanatoside C, digitoxigenin and digoxigenin.

4. *The view that binding of the glycosides in blood by serum protein* (albumin in particular) conditions their availability in the animal needs to be re-examined. Experiments with radioactive digitoxin indicate that there appears to be a greater degree of correlation between the lipid content of serum protein fractions and the "binding" of digitoxin than that between the serum albumin and glycoside.

Animal studies reveal many differences in the quantitative excretion and metabolism of the digitalis glycosides between species and it is not possible to apply results obtained by animal experiments to man. It has been shown however that man excretes the same metabolites as are found in rat urine and a quantitative estimation of these metabolites in human urine is needed.

The glycoside most intensively studied is digitoxin. There is a good deal of evidence to indicate that the well-known cumulative effect of this substance compared with the other clinically important glycosides (digoxin, lanatoside C) can be partly attributed to its lipid solubility and partly to an entero-hepatic cycle. Although only 10 per cent of the total dose of digitoxin is excreted in the bile of rats during the 5 hours following an injection, a small continuous excretion may occur over a longer period as the glycoside or its metabolites are released from body tissues and organs. The digitoxin excreted in bile is most probably re-absorbed to a considerable extent while the more polar metabolites are less completely absorbed and are excreted in the feces. This would account for

the detection of digoxin rather than digitoxin in rat feces by Repke[118] after digitoxin administration. Evidence from rat studies also indicates that the less polar glycosides digoxin and lanatoside C are less cumulative than digitoxin because they are more readily excreted in bile and less likely to be re-absorbed from the intestine.

The radioactive studies of Okita *et al*[108] also indicate a cycling of digitoxin and its metabolites in humans. According to their results, re-absorbtion by the intestine of both digitoxin and its metabolites occurs, as the ileum and ileal contents contain a lower concentration of labeled compounds that does the jejunum. Also a decrease in the metabolite: digitoxin ratio during passage of the labeled compounds from the small to the large intestine appears to indicate a greater degree of absorption of metabolite than digitoxin. Little is known however about the polarity of the metabolites. The kidney continually removes small amounts of the metabolic products and lesser amounts of the unchanged glycoside, so that in this study (restricted to 3 patients only) approximately 70 per cent of the original dose of digitoxin was excreted in the urine as metabolic products and 8 per cent as unchanged digitoxin. Fecal excretion accounted for about 9 per cent of the dose as unchanged drug and 8 per cent of metabolic products. The kidney is undoubtedly the most important organ in the ultimate excretion of digitoxin.

In vitro experiments and isolated organ perfusions have provided evidence previously lacking (Friedman[46]) that most organs and tissues possess enzymes capable of inducing hydrolytic fission of the glycosides to produce the genin and release the sugar residues Some free genin can be detected in *in vitro* experiments, but the indications are that in the intact animal the genin so produced is conjugated with some unknown agent. This conjugation also occurs *in vitro*.

The hydroxylation of digitoxin also occurs *in vitro* in most tissues and organs, and the fact that this occurs in both rat and human blood and serum, indicates that relatively non-specific enzyme systems are involved in the reaction Although the liver is undoubtedly the most important organ in regard to metabolism, the role of other tissues and organs may be significant.

The facts that have emerged from a good deal of intensive study

of the fate of cardiac glycosides in animals have not helped very much in showing how these substances act in restoring the failing heart. Indeed this is not the main object of these studies. A good deal has been learned about the changes which are undergone by the glycosides in their passage through the body, although there is yet no knowledge of any formation of any non-steroidal metabolites or metabolites in which the unsaturated lactone ring of the glycosides is not intact. The study of the metabolites produced from the glycosides has not yet thrown any more light upon the relationship between chemical structure and activity in this group of drugs unless it is to confirm the specificity of the structure of the aglycone. The sugars may be disposed of, but the steroid portion of the molecule either remains intact or is only slightly modified by hydroxylation to act in what appears to be the same way as the parent glycoside.

With regard to other aspects of the problem of the action of cardiac glycosides, more attention needs to be given, as pointed out by Friedman,[44] to the development and use of new tools to study the changes induced by the glycosides in the intact cell.

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